Investigations on the rapid transbilayer movement of phospholipids in biogenic membranes

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Abbreviations

ABC	ATP binding cassette
AEC	anion exchange chromatography
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
APLT	aminophospholipid translocase
BSA	bovine serum albumin
CL	cardiolipin
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid
ePC	egg phosphatidylcholine
ER	endoplasmic reticulum
HEPES	4-(hydroxyl)-1-piperazine-ethanesulfonic acid
IEC	ion exchange chromatography
IIMV	inverted inner membrane vesicle
LPP	major outer membrane protein
LPS	lipopolysaccharide
MDO	membrane-derived oligosaccharide
MDR	multi drug resistance
M-C6-NBD-PC	1-myristoyl-2-[6-(NBD)aminocaproyl]phosphatidyl-choline
M-C6-NBD-PE	1-myristoyl-2-[6-(NBD)aminocaproyl]phosphatidyl-ethanolamine
M-C6-NBD-PG	1-myristoyl-2-[6-(NBD)aminocaproyl]phosphatidyl-glycerol
NBD	4-nitrobenzo-2-oxa-1,3-diazole
N-DP-NBD-PE	N-NBD-dipalmitoyl-phosphatidylethanolamine
NEM	N-ethylmaleimide
PA	phosphatidic acid
PC	phosphatidylcholine
P-C6-NBD-PS	1-palmitoyl-2-[6-(NBD)aminocaproyl]phosphatidylserine

PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PMSF	phenylmethylsulfonyl fluoride
pss	phosphatidylserine synthetase
QF	flow through of the anion exchange column
QE	eluate of the anion exchange column
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
TE	triton extract
TEA	tri ethanol amine
TNBS	2,4,6-trinitrobenzene sulfonic acid
TLC	thin-layer chromatography
Tris	tris(hydroxymethyl)aminoethane

1 Introduction

All cells are surrounded by a plasma membrane consisting of two layers (leaflets) of amphipathic lipid molecules. This so-called lipid bilayer comprises a hydrophobic inner region formed by the hydrophobic tails of the lipid molecules and a polar outer region composed of the head groups of lipids. This lipid bilayer forms the physical barrier between the aqueous cytoplasm and the surrounding. Within the lipid bilayer proteins are embedded ("fluid-mosaic" model by Singer and Nicolson (Singer and Nicolson, 1972)). The proteins traverse the two leaflets (integral or intrinsic proteins) or are attached to membrane (peripheral proteins). In addition to the ubiquitous plasma membrane, eukaryotic cells contain subcellular membranes creating different intracellular compartments in which highly specific biochemical processes can be maintained and regulated. For the specific function of each compartment, distinct sets of lipids and proteins are essential. Moreover, lipids have to adopt the correct distribution over the two membrane leaflets. For example, in the plasma membrane of bacteria phospholipids are synthesized on the cytoplasmic leaflet of the plasma membrane. To ensure balanced growth and thus, stability of the biogenic membrane, half of the newly synthesized lipids must move to the opposing leaflet. A similar process must occur in the endoplasmic reticulum (ER) of eukaryotic cells. Newly synthesized lipids are initially located in the cytoplasmic leaflet of the ER but must flip across the ER to populate the exoplasmic leaflet to allow balanced membrane growth.

Furthermore, the plasma membrane of eukaryotic cells displays an asymmetric lipid distribution with the majority of aminophospholipids in the cytoplasmic leaflet and choline-containing phospholipids in the exoplasmic leaflet. Because this lipid asymmetry does not correspond to the asymmetry of lipid synthesis or hydrolysis, it must be formed and maintained by specific mechanisms that control lipid movement across the bilayer.

In protein-free model membranes, movement of most phospholipids from one leaflet to the other - the so-called flip-flop - is very slow, with half-times in the order of days (Eastman, et al., 1991; Kornberg and McConnell, 1971). The reason for the very slow flip-flop is the thermodynamically unfavorable transfer

of the hydrophilic head-group of a lipid molecule through the hydrophobic core of the lipid bilayer. Nevertheless, phospholipid transbilayer movement must occur at a considerable faster rate in membranes of living cells. This has led to the idea that lipid flip-flop is protein-mediated. The identification and characterization of the protein machinery involved in lipid flip-flop is a major challenge in current biology.

In the first chapter, an overview about the phospholipid flip-flop in eukaryotic cells is presented. Subsequently, the consequences of transport for function and structure of the originating and target membranes are discussed. Since this thesis focuses on the mechanisms of phospholipid flip-flop across the inner membrane in *Escherichia coli* (*E.coli*), the present knowledge about the composition and functions of phospholipids in the *E.coli* envelope are summarized and the known phospholipid transport processes in bacteria will be discussed. In the last paragraph of this chapter, a number of techniques and methods used for investigations in transmembrane distribution and movement of (phospho)lipids will be described. Finally, the aims of the studies presented in this thesis are summarized.

1.1 Phospholipid transmembrane movement in eukaryotic cells

In eukaryotic cells, the phospholipid transbilayer distribution is specific for various subcellular membranes and seems to be regulated by specific membrane proteins (for a review see e.g. (Pomorski, et al., 2001)).

The plasma membranes of eukaryotic cells have a clearly defined transbilayer phospholipid distribution. The aminophospholipids phosphatidylethanolamine (PE) and phosphatidylserine (PS) are highly enriched in the cytoplasmic leaflet compared to the exoplasmic leaflet. This asymmetry is generated by a not yet identified aminophospholipid translocase (APLT) that uses hydrolyses of ATP to translocate aminophospholipids from the exoplasmic to the cytoplasmic leaflet (Seigneuret and Devaux, 1984; Tilley, et al., 1986). The APLT activity is diminished after modification of plasma membrane proteins with sulfhydryl reagents like NEM, in the presence of vanadate and after elevation of the intracellular calcium concentration, which leads to a randomization of phospholipids across the plasma membrane leaflets in each case (Bitbol, et al., 1987; Herrmann, et al., 1989; Williamson, et al., 1992). Furthermore, in human erythrocyte ghosts, it has been shown that PS translocase activity and a Mg^{2+}-ATPase activity are properties of the same protein (Beleznay, et al., 1997). In a variety of subcellular membranes like chromaffin granules, clathrin-coated vesicles and cholinergic vesicles from *Torpedo* electric organ Mg^{2+}-ATPases of unknown function have been discovered. They share several properties with APLT, e.g. stimulation by PS and inhibition by vanadate and NEM. (Xie, et al., 1989; Yamagata and Parsons, 1989; Zachowski, et al., 1989). Although many attempts have been made to identify the APLT (Auland, et al., 1994; Morrot, et al., 1990; Zimmerman and Daleke, 1993), its molecular identity remains unclear. The asymmetric distribution of PS and PE across the plasma membrane is dissipated by the action of a putative, Ca^{2+}-activated scramblase (Bevers, et al., 1999; Comfurius, et al., 1990). The resulting exposure of PS and PE in the outer leaflet leads to various cellular responses, e.g. recognition by macrophages (Chang, et al., 2000; Fadok, et al., 1992; Verhoven, et al., 1995) or blood coagulation (Solum, 1999).

A second member of ATP-dependent lipid transporter, the ATP-binding cassette (ABC) transporter family, was identified in studies, originally related to multidrug resistance (MDR) in cancer cells. ABC proteins transport a broad spectrum of structural unrelated substrates (Bosch and Croop, 1996). Studies on MDR1 P-glycoprotein (MDR1-Pgp) showed that MDR1-Pgp affects the transverse distribution of endogenous PS and PE (Bosch, et al., 1997; Pohl, et al., 2002). Unlike MDR1-Pgp, MDR3-Pgp is thought to regulate the secretion of PC into the bile (Elferink, et al., 1997; Smit, et al., 1993).

The ER membrane, in contrast to the plasma membrane, as a biogenic membrane, is assumed to have a symmetric lipid distribution. This transmembrane distribution and movement is thought to be mediated by a non-specific protein. It has been proposed that as a result of the activity of this protein, all phospholipids abundant in the microsomal membrane are continuously randomized and therefore, are equally distributed across the bilayer (Herrmann, et al., 1990; Williamson and Schlegel, 1994). It has been shown that phospholipids rapidly equilibrate over rat liver microsomal membranes by facilitated diffusion (Bishop and Bell, 1985; Buton, et al., 1996; Herrmann, et al., 1990). The measured translocation rates depended on the type of phospholipid analogue, temperature and differ slightly between methods used to assay this process (reviewed in (Menon, 1995)). Buton *et al.* reported a characteristic translocation half-time of fluorescent short-chain phospholipid analogues (see chapter 1.3.2) across the microsomal membrane of ~25 s (Buton, et al., 1996). This rapid movement was determined to be bi-directional, partially protease- and NEM-sensitive (Buton, et al., 1996). Buton and co-workers used a combined BSA extraction and filtration assay, which was limited in time resolution to properly monitor the very fast phospholipid flip-flop (Buton, et al., 1996). In a more recent study, Herrmann and co-workers improved the measurements of ultra-fast translocation processes (Marx, et al., 2000). They significantly enhanced the time resolution due to a combination of the classical BSA back-exchange assay with the stopped-flow method, which was intensively used and further optimized in this thesis. Marx *et al.* found that fluorescent and spin labeled, short-chain phospholipid analogues rapidly redistributed between the leaflets of microsomes with half-times of

62-148 s and 8-16 s, respectively (Marx, et al., 2000). Collectively, these studies revealed that the fast transbilayer movement in the ER is a protein-mediated, bi-directional process without phospholipid specificity. However, whether specific proteins are required for this phospholipid flip-flop remains to be elucidated. Recent studies discussed the possibility that transmembrane stretches of membrane proteins facilitate the phospholipid translocation (Kol, et al., 2001; Kol, et al., 2003; Kol, et al., 2003). In an attempt to identify the proteins involved in the rapid transmembrane movement of phospholipids across the ER, Menon and colleagues reconstituted detergent extracts and fractions of microsomal membranes separated by glycerol gradient centrifugation into proteoliposomes (Menon, et al., 2000). This approach yielded a chromatographic fraction of enhanced transport activity, which sedimented at 3.8 S in the glycerol gradient. However, they were not able to isolate a specific protein that was responsible for the translocation.

1.2 The architecture and dynamic of the bacterial envelope

1.2.1 The membrane organization of *E.coli*

With regard to the cell membrane structure, bacteria can be divided in two classes: Gram-positive and Gram-negative bacteria. Gram-positive bacteria like *Bacilli* contain only an inner membrane and a peptidoglycan layer. Gram-negative bacteria such as *E.coli* have an inner and outer membrane, with an aqueous compartment between the two membranes - the periplasm. This compartment harbors the peptidoglycan layer.

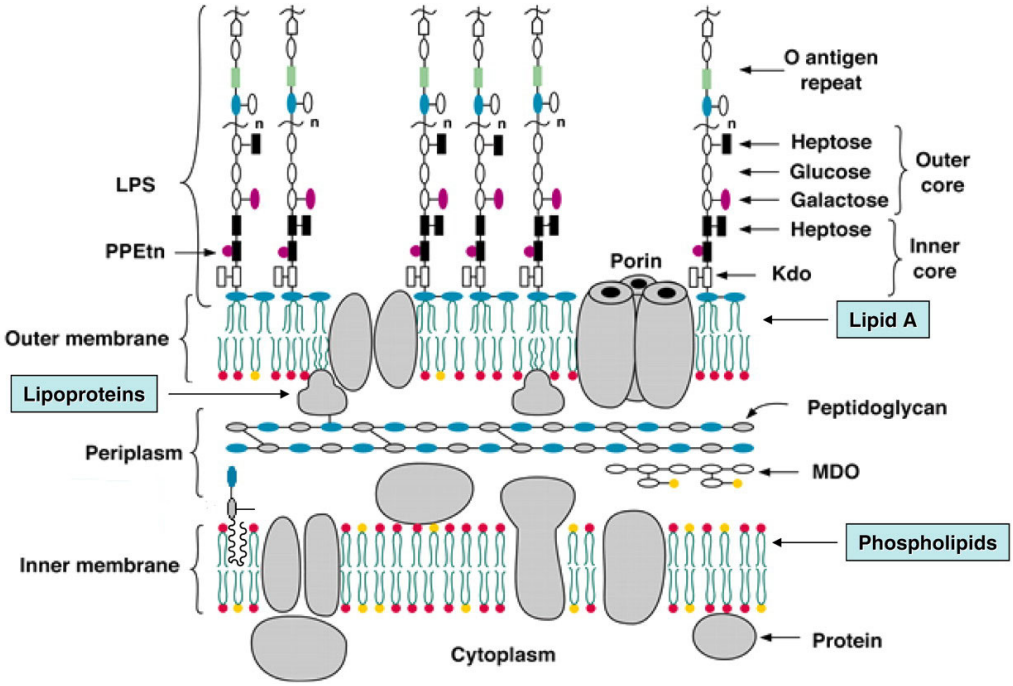


Figure 1: Schematic representation of the E.coli envelope adapted from Raetz and Withfield with minor modifications (Raetz and Whitfield, 2002). Abbreviations: LPS, lipopolysaccharide; MDO, membrane-derived oligosaccharides; Kdo, 3-deoxy-d-manno-oct-2-ulosonic acid; PPEtn, phosphoethanolamine

The outer membrane forms a semipermeable border of the *E.coli* cell to its environment (Figure 1). It consists of lipopolysaccharide (LPS), glycerophospholipids and proteins. The outer membrane organization is highly asymmetric. The inner leaflet of the outer membrane is exclusively populated by phospholipid molecules (predominantly PE), while the outer leaflet exclusively contains LPS (Osborn, et al., 1972). LPS is a complex phospholipid with a non-repeating “core” oligosaccharide and a distal polysaccharide. The hydrophobic

anchor, lipid A or endotoxin, is a glucosamine dimer with six acyl chains attached. The peripheral polysaccharide chain contains distinct types of sugars. *E.coli* mutants lacking several sugar residues in the inner core of LPS (deep rough mutants, (Nikaido and Vaara, 1985)) are more sensitive for penetration of hydrophobic macromolecules (Nikaido and Vaara, 1985). Furthermore, deep rough mutants contain additional phospholipids in the outer leaflet, which is thought to contribute to enhanced permeability. LPS, in particular lipid A, plays an important role in activation of innate immune responses e.g. by. macrophages (Raetz and Whitfield, 2002).

The most abundant outer membrane proteins are integral pore forming proteins with a β-barrel structure, so-called porins (Nikaido and Vaara, 1985). These proteins allow non-specific passage of small hydrophilic molecules with a molecular mass of up to 600 Da (Decad and Nikaido, 1976). Several of the outer membrane β-barrel proteins transport water-soluble molecules like sugars and nucleotides in a more regulated manner. The outer membrane also contains a variety of peripheral and integral non-porin proteins with different functions e.g. receptors for vitamins (Nikaido and Vaara, 1985) and proteins involved in membrane and cell shape stability, like protein A (OmpA) (Pautsch and Schulz, 2000). The outer membrane also contains proteins with enzymatic activities, like OmpT and OMPLA (Dekker, et al., 1999; Luirink, et al., 1986; Pugsley and Schwartz, 1984; Stathopoulos, 1998; Vandeputte-Rutten, et al., 2001).

The periplasm (Figure 1) harbors the murein sacculus (peptidoglycan layer), which is a polymer of muropeptides (Weidel and Pelzer, 1964). The muropeptide polymer protects the cells from lysis in a hypotonic environment and is an important factor in maintaining the cell shape (Rogers, et al., 1980). The peptidoglycan layer is bound to the outer membrane by the major outer membrane protein (LPP) (Braun and Rehn, 1969; Braun and Wolff, 1970) via peptide bonds. OmpA is also involved in attaching the peptidoglycan wall to the outer membrane.

Additionally, to a number of proteins involved in transport, detoxification, metabolic and catabolic processes (Beacham, 1979; Tam and Saier, 1993), the periplasm contains membrane-derived oligosaccharides (MDO). These sugar

chains are substituted with phosphoethanolamine, phosphoglycerol and O-succinyl ester (Kennedy, et al., 1976) and believed to play a role in osmotic regulation.

The inner membrane of *E.coli* separates the cytoplasm from the extracellular environment. It is the side of many essential processes, like nutrient uptake, oxidative phosphorylation, transport processes and export of toxic substances as well as metabolic products. Most of the proteins in *E.coli* that are involved in protein and lipid biosynthesis are associated with this membrane. Especially the catalytic active sites of proteins involved in phospholipid synthesis are faced to the cytosolic side of the membrane (Huijbregts, et al., 2000).

1.2.2 Glycerophospholipids in *E.coli*

The major phospholipids of *E.coli* are PE and phosphatidylglycerol (PG), which make up ~80% and 15% of the total phospholipid content, respectively. The third major phospholipid is cardiolipin (CL) with an abundance of about 5%.

Phospholipid synthesis starts in the cytosol with the generation of glycerol-3-phosphate (G3P) (Kito and Pizer, 1969). G3P is acylated in the sn-1 and sn-2 position and the resulting phosphatidic acid (PA) is immediately converted to CDP-glyceride (Sparrow and Raetz, 1985), as evident by the finding that PA is present only in trace amounts (~1%) in the plasma membrane of *E.coli*. CDP-glyceride is the precursor for the synthesis of PE, PG and CL. The first step in the synthesis of PE is the reaction of CDP-glyceride with serine, catalyzed by the phosphatidylserine synthetase (pss). PS is rapidly decarboxylated to PE by the PS decarboxylase. PE, the most abundant phospholipid in *E.coli*, is a zwitterionic molecule at physiological pH, due to the protonated amino group and the dissociated phosphate group.

The synthesis of the acidic phospholipid PG, the second major phospholipid in *E.coli*, starts with the formation of phosphatidylglycerolphosphate (PGP). The substrates of this reaction are CDP-glyceride and G3P. PGP is converted to PG by the enzyme PGP phosphatase.

CL is formed by the condensation of two molecules of PG (Hirschberg and Kennedy, 1972; Tunaitis and Cronan, 1973) by the enzyme CL synthetase.

1.2.3 Phospholipid movement across bacterial membranes

In Gram-negative bacteria, phospholipids have to move from the site of their synthesis, the cytoplasmic leaflet of the inner membrane, to the periplasmic leaflet. In addition, phospholipids have to be transported to the inner leaflet of the outer membrane. Both processes have been studied in *in vivo* and *in vitro* systems (for a recent review see (Huijbregts, et al., 2000)). The movement of phospholipids between the inner and the outer membrane was first shown by Osborn and co-workers (Osborn and Munson, 1974). They demonstrated with pulse labeling studies that PE was synthesized in the inner membrane of *Salmonella typhimurium* and finally transported to the outer membrane. Moreover, radiolabeled PS, introduced in the outer membrane, was shown to be rapidly transported to the inner membrane, where it becomes accessible to the enzyme PS decarboxylase and transformed to PE within five minutes (Jones and Osborn, 1977; Jones and Osborn, 1977). The resulting PE was transported back to the outer membrane. In the same study, it was shown that the transport of phospholipids in *S. typhimurium* was head-group independent, since not only the major phospholipids (PE, PG and CL) were transported, but in addition, the not naturally occurring phospholipid PC was also transported to the inner membrane of *S. typhimurium*.

In Gram-negative bacteria, membrane contact sides between inner and outer membrane, so called Bayer's bridges (Bayer, 1991), have been suggested to mediate intermembrane lipid transport. Additionally, in *E.coli* a rapid bi-directional transport of phospholipids between the inner and the outer membrane was observed on whole cells (Donohue-Rolfe and Schaechter, 1980; Langley, et al., 1982).

The first investigations on transbilayer movement of phospholipids in bacteria has been carried out on Gram-positive bacteria (Rothman and Kennedy, 1977). Rothman and Kennedy observed that the translocation of newly synthesized PE from the inner to the outer leaflet in *Bacilli* occurred with a half-

times of 1.5-3 min at 37°C (Rothman and Kennedy, 1977; Rothman and Kennedy, 1977). A more recent study (Hrafnsdottir, et al., 1997) showed that short-chain, fluorescent labeled phospholipid analogues translocated rapidly across the *Bacillus megaterium* membrane with a half-time of ~30 s at 37°C. This transport was demonstrated to be protease sensitive but not head-group dependent. Transbilayer movement of phospholipids was studied with the use of reconstituted, transport-competent proteoliposomes derived from detergent-solubilized *Bacillus subtilis* plasma membranes (Hrafnsdottir and Menon, 2000). The resulting proteoliposomes were shown to be capable of transporting a short-chain, water soluble analogue of PC (half-time about one minute). To prove that the short-chain, water soluble PC analogue reflects the behavior of endogenous phospholipids, a more natural long-chain phospholipid dipalmitoyl-PC (DPPC) was reconstituted into proteoliposomes and the extent of hydrolysis by the phospholipase A_2 (PLA_2) was measured (Hrafnsdottir and Menon, 2000). Indeed, the extent of hydrolysis was shown to be a function of the protein/phospholipid ratio reconstituted into proteoliposomes derived from *B. subtilis*. This indicates that the short-chain as well as the long-chain phospholipid analogues were transported across the vesicle membrane by proteins.

Pulse labeling studies on separated inner and outer membrane fractions from *E.coli* demonstrated that newly synthesized PE reached the outer membrane within 2.8 min. The transport of anionic phospholipids had a half-time of less than 30 s (Donohue-Rolfe and Schaechter, 1980; Langley, et al., 1982). Investigations on inverted inner membrane vesicles (IIMV) from *E.coli* also demonstrated a rapid transbilayer movement of phospholipids across the vesicle membrane (Huijbregts, et al., 1996). Utilizing short-chain fluorescent analogues of phospholipids, Huijbregts *et al.* showed that exogenously added analogues rapidly flip across the inner membrane of *E.coli* with a half-time about seven minutes at 37°C. This transport was temperature dependent, bi-directional and not influenced by treatment with sulfhydryl reagents or proteinase K, nor by the presence of ATP or a pH gradient across the membrane of IIMV (Huijbregts, et al., 1996). Huijbregts, *et al.* also studied transmembrane movement of endogenously synthesized phospholipids across the inner membrane of *E.coli* (Huijbregts, et al., 1998). Radioactive labeled PE was biosynthetically introduced into IIMV from

PE-deficient *E.coli* strain AD93 by reconstitution with the enzyme pss and the addition of wild-type lysate, metabolic substrates and [^{14}C]serine. Another approach utilized right-side out vesicles, in which the active site of pss is situated in the lumen of the vesicles. Under these circumstances, the PS conversion took place in the lumen of the vesicles by reconstitution and the appearance of PE on the outer leaflet was measured. Both approaches demonstrated that the redistribution of newly synthesized radiolabeled PE occurred with a half-time of less than one minute. However, these earlier studies did not demonstrate a strong requirement for protein in the translocation process or lacked the time resolution to measure an accurate translocation rate. Furthermore, the molecular basis of the putative protein dependent mechanism of phospholipid flip-flop is still unknown.

Only little is known about the transverse distribution of phospholipids in bacterial membranes. In the plasma membrane of the Gram-positive bacterium *Micrococcus luteus* the distribution of PG and CL was studied using photoreactive lipid analogues (de Bony, et al., 1989). A slight asymmetric distribution of PG with about 60% of the PG in the outer leaflet was found. CL was equally distributed between the two leaflets. However, in a later report it was suggested, that this distribution strongly depends on cell growth and division (Welby, et al., 1996). When Huijbregts and colleagues investigated the transbilayer distribution of phospholipids in IIMV and right-side out vesicles, they detected an asymmetric transbilayer distribution of radiolabeled, newly synthesized PE in the inner membrane of 35% in the cytoplasmic and 65% in the periplasmic leaflet (Huijbregts, et al., 1998).

1.3 Methods for the characterization of transmembrane distribution and movement of phospholipids in biological membranes

A variety of techniques are used to investigate the phospholipid transbilayer distribution and movement in model membranes and biomembranes such as plasma membranes or cellular organelles. In the past, chemical reagents or enzymes for altering endogenous phospholipids or high affinity probes, which react with distinct phospholipid species, were utilized. In the last two decades, lipid analogues, which mimic the physicochemical properties of their endogenous counterparts, evolved to be the most important tools to study the behavior of lipids.

1.3.1 Assays for the determination of transmembrane movement and distribution of endogenous phospholipids

One of the first methods used to determine the transbilayer distribution of phospholipids was the modification of endogenous phospholipids by phospholipases A_2 (PLA_2) and C (PLC). Endogenous phospholipids located in the outer monolayer of membranes, such as the plasma membrane of erythrocytes (Dolis, et al., 1996; Roelofsen and Zwaal, 1976) or the plasma membrane of prokaryotes (Nanninga, et al., 1973), were treated with PLA_2 or PLC. Subsequently, the products were analyzed by e.g. chromatographic techniques. This invasive method has a number of shortcomings. Phospholipase treatment can lead to the release of lyso lipids and free fatty acids from the membrane (Nanninga, et al., 1973), and therefore, may induce transmembrane movement of phospholipids and their derivatives or perturbations of the membrane organization. Furthermore, these assays suffer from the limited time resolution.

Other approaches are based on modifications of the head group or fatty acid of lipids by chemicals (reviewed in (Op den Kamp, 1979)), like trinitrobenzene sulfonic acid (TNBS), isothionyl acetimidate, fluorescamine or anthracene (Welby, et al., 1996). TNBS reacts specifically with PE and does not permeate the membrane. It has been widely used for investigations on the transverse distribution of this phospholipid in both prokaryotic (Rothman and Kennedy, 1977) and eukaryotic cells (Bonsall and Hunt, 1971; Cerbon and Calderon, 1991;

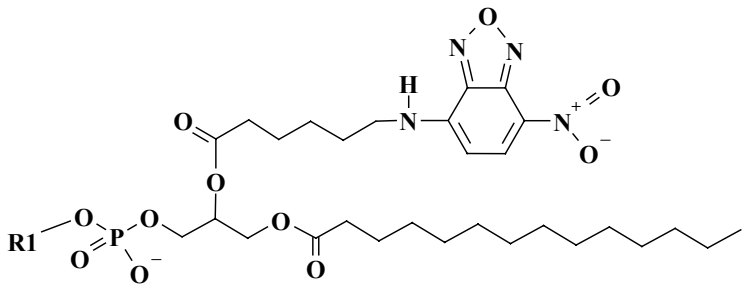
Fontaine, et al., 1980; Marinetti and Love, 1976; Musters, et al., 1993; Sandra and Cai, 1991). Another chemical reagent, which has been utilized to determine the distribution and transmembrane movement of PG across vesicle membranes is the α-diol group oxidizing periodate (de Bony, et al., 1989; Hope, et al., 1989; Huijbregts, et al., 1997). But permeation of periodate through distinct membranes leads to inaccuracies in the quantification of the PG distribution across the membrane (Huijbregts, et al., 1997).

Non modifying techniques were also applied, such as the annexin V approach. Annexin V is a protein, which is able to non-covalently bind to various phospholipids in a calcium dependent manner but with a clear preference for PS (Swairjo and Seaton, 1994). Annexin V does not penetrate the membrane. However, annexin V is only appropriate for the determination of the exposure or presence of phospholipids rather than for the assessment of transmembrane movement and distribution of phospholipids.

Lipid transfer proteins, which can transfer lipids between two membranes (Wirtz, 1991) have also been used to study the distribution of phospholipids in membranes (van Meer, 1989; Wirtz, 1991).

1.3.2 Assays for the determination of transmembrane movement and distribution of phospholipid analogues

Substantial progress in the study of transmembrane distribution and transbilayer movement of phospholipids across membranes has been made with the introduction of phospholipid analogues (Kornberg and McConnell, 1971; McIntyre and Sleight, 1991). Fluorescent and spin labeled analogues have been extensively used in many studies. One of the naturally occurring long-chain fatty acids (usually in the sn-2 position) of the phospholipid is substituted by an acyl chain (commonly 4-12 carbon atoms long) carrying a fluorescent (e.g. N-(4-nitrobenzo-2-oxa-1,3-diazole) (NBD)) or paramagnetic (e.g. 4-doxyl) reporter group. This replacement results in a slight change of the physicochemical properties of the analogue. The shorter fatty acid in the sn-2 position makes the analogues slightly more water-soluble compared to their endogenous counterparts. This is advantageous for rapid incorporation of these analogues into



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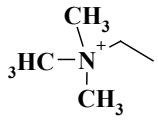
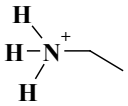
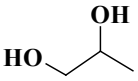


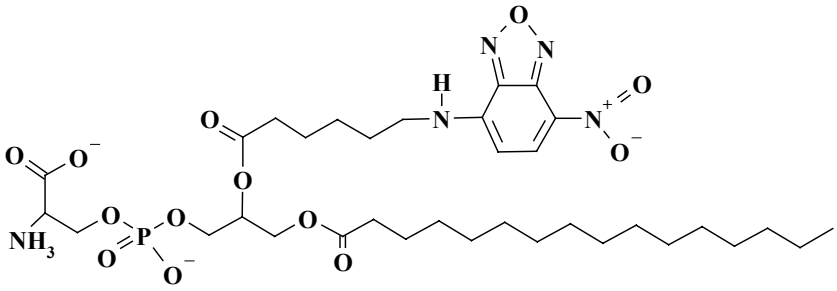
1-Myristoyl-2-[6-(NBD)aminocaproyl]phosphatidylcholine
(M-C6-NBD-PC)



1-Myristoyl-2-[6-(NBD)aminocaproyl]phosphatidylethanolamine
(M-C6-NBD-PE)



1-Myristoyl-2-[6-(NBD)aminocaproyl]phosphatidylglycerol
(M-C6-NBD-PG)



1-Palmitoyl-2-[6-(NBD)aminocaproyl]phosphatidylserine (P-C6-NBD-PS)

Figure 2: Structures of fluorescent short-chain phospholipid analogues used in the course of this thesis.

(bio)membranes, since the short-chain analogues spontaneously incorporate into membranes. Their transmembrane distribution and movement can be monitored either by fluorescence spectroscopy, fluorescence microscopy for fluorescent labeled or by EPR spectroscopy for spin labeled analogues. Furthermore, the reporter groups can be chemically modified either by conversion of spin labeled analogues into diamagnetic species using ascorbate (Kornberg and McConnell, 1971) or by turning fluorescent analogues into non-fluorescent derivatives using dithionite (Huijbregts, et al., 1996; McIntyre and Sleight, 1991; Pomorski, et al., 1994).

Short-chain (up to six carbon atoms with the fluorophore attached) phospholipid analogues (Figure 2) are extractable by e.g. BSA (Haest, et al., 1981). That makes the so-called BSA back-exchange assay a powerful tool in the investigation of the transbilayer movement and distribution of phospholipids. The time resolution of this assay is higher than of assays that utilize enzymes for the determination of the transmembrane distribution of endogenous phospholipids. However, for some membrane systems it has been shown that BSA is not capable of quantitatively extracting short-chain phospholipid analogues. In such cases, time-consuming centrifugation steps are required (Pomorski, et al., 1996).

A general concern in these studies is to what extent these analogues mimic the behavior of endogenous phospholipids in membranes. Since their sn-2 acyl are shorter than the respective acyl chains of the native counterparts, and therefore, possess some aqueous solubility, analogues are capable of spontaneous monomeric exchange between (intracellular) membranes of nucleated cells (Bai and Pagano, 1997; Kean, et al., 1993; Martin and Pagano, 1987; Nichols and Pagano, 1982). Consequently, as far as nucleated cells are concerned, the transmembrane equilibrium distribution of analogues does not provide a quantitative measurement of the actual steady-state distribution of the corresponding endogenous phospholipids. However, it has been shown that the transmembrane equilibrium distribution of analogues qualitatively reflects the distribution of endogenous phospholipids (Bratton, et al., 1997; Verhoven, et al., 1995).

Another concern is the presence of a reporter group that might influence the kinetics and the extent of transmembrane movement of the analogue. Tilley *et al.* obtained kinetic data for phospholipid translocation of spin-labeled analogues very similar to data obtained with radioactive, long-chain phospholipids (Tilley, et al., 1986). As the doxyl group is a comparatively small reporter group, the behavior is likely to resemble endogenous phospholipids with respect to the transmembrane movement.

The NBD moiety, a fluorescent reporter group, is much bulkier and more polar than the doxyl group. It was found that this group diminishes the affinity of PE analogues to the APLT (Colleau, et al., 1991). Additionally, Chattopadhyay and London, who used fluorescence quenching by spin-labeled phospholipids,

concluded that presumably the polarity of the NBD group results in “loop back” of the reporter group to the membrane surface (Chattopadhyay and London, 1987). Indeed, as shown by dithionite fluorescence assay, long-chain analogues react more favorably with dithionite, indicating a better accessibility of the probe by dithionite present in the aqueous phase (Huster, et al., 2001; Huster, et al., 2003). It cannot be excluded that the bending of the sn-2 acyl chain might exert an influence on kinetic analysis.

However, short-chain, NBD-labeled analogues (Figure 2) have proven to be faithful analogues of their endogenous counterparts in a variety of membrane systems (Bratton, et al., 1997; Kean, et al., 1997; Marx, et al., 2000; Seigneuret and Devaux, 1984; Verhoven, et al., 1995). In spite of the concerns mentioned above, short-chain analogues are good tools that have proven to be useful for the identification of flippase proteins.

2 Scope

A long-standing problem in understanding the mechanism by which the phospholipid bilayer of biological membranes is assembled concerns how phospholipids flip back and forth between the two leaflets of the bilayer. This is important since the phospholipid biosynthetic machinery typically face the cytosol and deposit newly synthesized phospholipids in the cytosolic leaflet of biogenic membranes such as the ER of eukaryotic cells or the plasma membrane of prokaryotes. These lipids must be moved across the bilayer to ensure membrane growth and integrity. Transport does not occur spontaneously and is assumed to be facilitated by specific membrane proteins - the flippases.

During the last two decades, research efforts on protein-mediated phospholipid transmembrane movement in biogenic membranes focused on eukaryotic cells. Due to the inherent difficulties in the isolation of pure (sub)cellular membranes and in the genetic manipulation of eukaryotic cells, progress on the characterization, identification and purification of the flippase proteins of eukaryotic cells is slow.

The progress on the ultimate goal of identifying, purifying and possibly cloning the eukaryotic flippases is greatly enhanced by using much simpler organisms that are easily handled and manipulated on the genetic level. The Gram-negative bacterium *Escherichia coli* is a powerful model organism for investigations of biogenic membranes. *E.coli* does not contain subcellular membranes and therefore, a number of technical difficulties in studying transmembrane movement and distribution in eukaryotic systems, such as the combination of endocytosis and transbilayer movement in yeast (Kean, et al., 1993), are non-existent. Moreover, the protein content of a prokaryotic cell is much smaller than that of eukaryotic cells, hence, allowing an easier identification of an possible flippase. The genome of *E.coli* has recently been fully sequenced and was assumed to be close to a theoretical minimal genome necessary for ensuring survival of a cell. Since one of the genes within this small genome could be a flippase, the smaller number of total genes would make the identification of a flippase gene more likely.

For these reasons, *E.coli*, in particular the inner membrane of *E.coli*, was chosen as a model system for the characterization, identification and purification of flippases in biogenic membranes. Much progress has been made in elucidating the biogenesis and function of proteins in the membranes of *E.coli*. It has become apparent in the last three decades that lipids also play a role in a variety of processes within the *E.coli* cell and not only by forming a physical barrier. However, the knowledge about the mechanism by which phospholipids are transported across the membrane after synthesis and what the consequences are for the lipid distribution in the membrane, is still very limited. No biogenic membrane flippases have been identified so far, and there is a controversy as to whether proteins are involved at all, whether any membrane protein is sufficient, or whether non-bilayer arrangements of lipids facilitate phospholipid flip-flop.

The main objectives of this thesis were therefore (i) to introduce an assay with a high time resolution allowing the adequate quantitative characterization of the flip-flop in *E.coli*, (ii) to explore the transmembrane distribution of the phospholipids in the inner membrane of *E.coli*, (iii) to provide strong evidence for the hypothesis that the phospholipid flip-flop in the inner membrane of *E.coli* is protein dependent, has no head-group specificity and does not need energy input and (iv) to possibly, identify and purify the putative flippase of the plasma membrane of E.coli.

3 Material and Methods

3.1 Chemicals

M-C6-NBD-PE, M-C6-NBD-PG, M-C6-NBD-PC, P-C6-NBD-PE and P-C12-NBD-PE were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Egg PC, BSA, EDTA, NEM, ascorbic acid, PMSF, DTT, sodium dithionite were from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). TRIS, Na_2HPO_4, NaH_2PO_4, ammonium molybdate, sodium chloride, HEPES, Triton X-100 *analytical grade* and sucrose were purchased from Fluka Chemie AG (Buchs, Switzerland). Triton X-100 *ultra clean* was from Boehringer Mannheim GmbH (Mannheim, Germany). SM-Bio-Beads was obtained from Bio-Rad Laboratories (Hercules, USA). Fluka Chemie AG (Buchs, Switzerland) provided Polyacryl amide, TEMED, ammonium persulfate and SDS.

3.2 Preparation of inverted inner membrane vesicles from *E.coli*

Inner membrane vesicles were isolated from the *E.coli* wild type strain MG 1655. Cell growth and membrane isolation were essentially performed as described by Huijbregts *et al.* (Huijbregts, et al., 1996) with minor modifications.

A small volume of Luria Broth (LB) was inoculated with a pipette tip that was dipped into a deep freeze culture of MG 1655. This culture was grown at 37°C on a shaker at 140 U/min overnight (12 h-16 h). Nine milliliters of the overnight culture were diluted into three liters LB and grown to an optical density (OD_{660}) of 0.7-0.8 (late log phase). The cells were harvested by centrifugation in a type JLA 10.500 Beckman rotor (10 min, 2,000 g, 3°C) and washed with ice cold medium A (33 mM KH_2PO_4, 60 mM K_2HPO_4, 1.7 mM sodium citrate (hydrated) and 7.6 mM $(NH_2)_4SO_4$ pH 7.5). The sedimented cells were resuspended in 30 ml of buffer S (50 mM triethanolamine pH 7.5, 250 mM sucrose, 1 mM EDTA). DTT and PMSF were added to a final concentration of 1 mM and 0.375 mM, respectively. Cells were broken by 2-3 passages in a French press at a cell pressure of 1,100 psi. Subsequently additional DTT and PMSF were added to a final concentration 1 mM and 0.375 mM, respectively. Cell debris was removed

by centrifugation (2x for 10 min at 6000 g in a 45Ti Beckman rotor at 3°C). The outer membrane fraction was pelleted by centrifugation of the suspension in a SW 28 Beckman rotor for one minute on the maximum speed (165,000 g). To sediment the crude inverted inner membrane vesicles (IIMV), the supernatant was centrifuged at 165,000 g, for 90 min in a SW 28 Beckman rotor at 3°C. The resulting pellet was resuspended and homogenized with 15 strokes (one stroke corresponds to one down and one up move) in two milliliters ice cold buffer S and layered on top of a discontinuous sucrose gradient in buffer S0 (buffer S without sucrose) according to Osborn *et al.* 1972 (Osborn, et al., 1972). Subsequently, the gradients were centrifuged for 16 h to 18 h in a SW 40 Beckman rotor at 112,000 g at 3°C. Three bands were visible. The light band (corresponding to the purified IIMV, (Osborn, et al., 1972)) were collected and washed in 10 mM HPS (10 mM HEPES pH 7.5, 100 mM NaCl) at 165,000 g for 90 min and at 3°C (SW 28 rotor Beckman). Vesicles were resuspended in 10 mM HPS using a dounce homogenizer, quickly frozen in liquid nitrogen and stored at -80°C. IIMV suspensions were thawed immediately before use.

IIMV isolated as described above typically contained about 3 mg protein/µmol phospholipid. According to van Klompenburg *et al.* (van Klompenburg, et al., 1995), the vesicles are sealed and 100% inside-out.

3.3 Reconstitution of IIMV derived from *E.coli*

IIMV were solubilized and reconstituted according to the method described by Menon *et al.* (Menon, et al., 2000) and Hrafnsdóttir and Menon (Hrafnsdottir and Menon, 2000). In brief, the vesicle suspension was mixed with an equal amount of buffer DTEB (20 mM HEPES pH 7.5, 200 mM NaCl, 2% (w/v) Triton X-100). The mixture was incubated on ice for 30-60 min and subsequently centrifuged in a 70.1 Ti Beckman rotor at 175,000 g for 30 min to pellet unsolved proteins. The resulting triton extract (TE) was carefully collected and stored on ice until further reconstitution steps. The TE or chromatographic fraction was added to a solution of egg PC in buffer OTEB (10 mM HEPES pH 7.5, 100 mM NaCl, 1% (w/v) Triton X-100) with a final phospholipid concentration (after addition of the TE) of 4.5 µmol/ml. To incorporate fluorescent phospholipid analogues

symmetrically between the two leaflets of the reconstituted proteoliposomes the respective NBD-labeled phospholipids were added with a final concentration of max. two molpercent of total phospholipid content. For detergent removal 100 mg/ml (wet weight) SM-2 Bio-Beads® were added and gently rocked for three hours at room temperature. Subsequently, additional Bio-Beads were added (200 mg/ml wet weight), rocking was continued for additional two hours at room temperature. The mixture was transferred to 4°C and gently rocked for further 12 h-18 h. The resulting turbid suspension was separated from the beads using a glass Pasteur pipette and centrifuged for 45 min at 175,000 g, at 4°C (70.1 Ti Beckman rotor). The resulting pellet was resuspended in two milliliters 10 mM HPS, centrifuged again at 175,000 g for 45 min, 4°C, resuspended in the same buffer and homogenized with a dounce homogenizer (15 strokes) on ice.

3.4 Incorporation of NBD-labeled phospholipids into IIMV

The fluorescent phospholipid analogues (two molpercent of the total phospholipid content) in organic solvent were dried under a gentle stream of nitrogen. The resulting lipid film was dissolved in a small volume of ethanol (1% (w/v) of the final volume) and subsequently, suspended in 10 mM HPS. Two milliliters of the suspension were mixed with an aliquot of IIMV (12.5 µM final phospholipid concentration) and the fluorescence increase (excitation: 467 nm, emission: 540 nm, slid with: 4 nm) was monitored using an Aminco Bowman Series 2 spectrofluorometer (SLM Instruments Inc., Rochester, USA) for 1,600 s. All experiments were performed at room temperature.

3.5 The BSA back-exchange assay

NBD-labeled, short-chain lipid analogues were used to investigate the transmembrane movement and distribution of phospholipids across the inner membrane of *E.coli*. BSA is able to extract short-chain phospholipid analogues from given membranes. Back extraction of the phospholipid analogues by BSA from these membranes results in a fluorescence decrease because BSA-bound NBD-labeled phospholipid explores a different polarity with respect to

membranes. Extraction of fluorescent labeled, short-chain phospholipids can therefore be directly monitored by the fluorescence decrease.

3.5.1 Extraction of fluorescent labeled phospholipid analogues by BSA – cuvette experiments

To measure the rapid transbilayer movement of phospholipid analogues the BSA back-exchange assay was used. An aliquot of IIMV, proteoliposomes or liposomes labeled with max. two molpercent of total phospholipid content either M-C6-NBD-PC, -PE, -PG or P-C6-NBD-PS was diluted into two milliliters of 10 mM HPS. The suspension was placed in a quartz cuvette and the fluorescence was monitored (excitation: 467 nm, emission: 540 nm, slid with: 4 nm) using an Aminco Bowman Series 2 spectrofluorometer. After a constant fluorescence baseline was obtained (usually after 30 s), 20 µl of BSA in 10 mM HPS (20% w/v)) were added and the fluorescence decrease was measured for at least ten minutes at room temperature until a plateau of fluorescence intensity was reached.

3.5.2 The stopped-flow assay

All stopped-flow measurements were performed at room temperature. The time-dependent BSA back-exchange of NBD-labeled phospholipids was monitored by mixing the labeled IIMV, proteoliposomes or liposomes with 2% (w/v) BSA (final concentration) in 10 mM HPS using a stopped-flow accessory (RX 1000, Applied Photophysics, Leatherhead, UK) linked to an Aminco Bowman Series 2 spectrofluorometer. The dead time of mixing the two reactants amounts to about ten milliseconds. Extraction of analogues from membranes by BSA was followed by the decrease of fluorescence intensity. Fluorescence was recorded for 300 s at a time resolution of 0.2 s or 0.5 s, excitation wavelength λ_{ex}=467 nm, emission wavelength λ_{em}=540 nm, slit widths 4 nm. For each preparation, five or more kinetic traces were recorded and averaged for kinetic analysis (see 3.14). However, scattering of (non-labeled) liposomes, IIMV and IIMV-derived proteoliposomes was significant. Therefore, traces were corrected to compensate for the light scattering contribution to the amplitude of the signal.

3.6 The dithionite assay

Alternatively to the BSA assay, where the fluorescence decrease due to extraction of phospholipid analogues by BSA was monitored, a chemical fluorescence quenching assay was used to characterize the flip-flop of fluorescent phospholipid analogues. In this assay, the fluorescence is quenched by the chemical reaction of non-permeable dithionite ($S_2O_4^{2-}$) with the NBD-group of the phospholipid analogue (Figure 3), resulting in a non-fluorescent NBD-lipid derivative (McIntyre and Sleight, 1991). When unilamellar vesicles containing NBD-labeled phospholipids are mixed with dithionite, only the fluorescent lipids located on the outer leaflet of the vesicle bilayer are reduced.

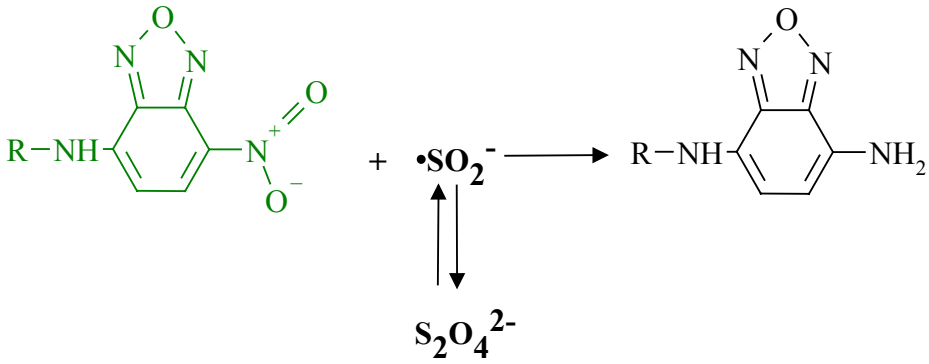


Figure 3: Chemical reaction of dithionite with the fluorescent NBD-group. The fluorescence of NBD is quenched by generation of a non-fluorescent derivative due to chemical interaction of the dithionite radical with the nitroxide group of the NBD-molecule.

To measure the transmembrane movement of fluorescent phospholipid analogues essentially the same experimental set-up as outlined in chapter 3.5 for the BSA back-exchange assay was used. Briefly, when performing cuvette experiments an aliquot of proteoliposomes derived from IIMV or chromatographic fractions from IIMV (see chapter 3.7) containing 0.5 mol% of the appropriate fluorescent analogue with respect to the total phospholipid content was suspended into two milliliters 10 mM HPS. Subsequently, the mixture was placed in a quartz cuvette and the fluorescence was monitored until a stable baseline was obtained. Then, freshly prepared dithionite in 40 mM Tris pH 8.0 was added to a final concentration of 10 mM and the fluorescence decay was measured for 600 s. Subsequently, the vesicles were disrupted by adding 1% (w/v)

Triton X-100 (final concentration) to test whether the concentration of dithionite was sufficient to quench the fluorescence completely.

When carrying out stopped-flow measurements, aliquots of the appropriate fluorescent labeled vesicles and 20 mM dithionite in 40 mM Tris pH 8.0 were mixed using the stopped-flow device and the resulting fluorescence decrease was measured.

All experiments were performed at 22°C to minimize the penetration of dithionite. The instrumental parameters used for both cuvette and stopped-flow measurements are described in chapter 3.5.

3.7 Ion exchange chromatography

In this study, ion exchange chromatography (IEC) was used to enrich flippase activity in a distinct fraction. All chromatographic steps were carried out at room temperature. An aliquot TE (see 3.3) was diluted 1:5 in buffer Z (25 mM TEA pH 8.0, 10 mM NaCl). This suspension was placed on a 1 ml Hi Trap Q HP column (Amersham-Pharmacia Biotech) equilibrated with buffer C (25 mM TEA acetate pH 8.0, 10 mM NaCl, 0.2% (w/v) Triton X-100). The Hi Trap column was operated using a peristaltic pump (BioRad) with a flow rate of 0.5 ml/min. The column was washed with three milliliters buffer A (10 mM HEPES pH 7.5, 100 mM NaCl, 0.2%(w/v) Triton X-100) and the wash was pooled with the flow-through. Bound proteins were eluted with buffer D (buffer A containing 1 M NaCl). Samples of each fraction were dialyzed against 1.5 l 10 mM HPS for one hour at room temperature and subsequently, reconstituted into proteoliposomes as described in chapter 3.3.

After dialysis, the resulting proteoliposomes were assayed by the dithionite approach as described in chapter 3.6.

The flippase activities of the reconstituted fraction were calculated as follows: The activity A is the percent of the fluorescent intensity above the pure liposome control, where $F_{red,lip}$ is the normalized fluorescent intensity of the liposomes after dithionite treatment and $F_{red,prot}$ is given by the final fluorescent intensity of the proteoliposome sample.

$$A = \left[F_{red,prot} - F_{red,lip}\right] * 100\%$$

The specific activity (A^S) describes the activity (A) of the probes relative to the protein/phospholipid ratio (P/PL) in %*µmol*µg^{-1}.

$$A^S = A / \left[P / PL\right]$$

3.8 SDS-PAGE analysis

Gels and buffers were made and the gel electrophoresis was run according to Laemmli (Laemmli, 1970). Aliquots of proteoliposomes were delipidated by the procedure of Bligh and Dyer (Bligh and Dyer, 1959) before subjecting to gel electrophoresis. The samples were mixed with sample buffer and heated to 95°C for several minutes. Subsequently, the samples were applied to sodiumdodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). Finally, the gels were silver stained as follows: First, the gels were fixed for at least 30 min in 100 ml aqueous solution containing 30% (v/v) ethanol and 10% (v/v) acetic acid. Afterwards, the gels were incubated for 30 min in 100 ml of a solution of 30% (v/v) ethanol, 0.5% (v/v) glutaraldehyde, 0.2% (w/v) sodium thiosulfate and 0.5 M sodium acetate. Subsequently, the gels were washed with 100 ml *aqua dest.* for ten minutes. The wash was repeated twice. Then, the proteins were stained by incubation of the gel in the color solution containing 0.1% (v/v) silver nitrate and 0.02% (v/v) formaldehyde. Thereafter, the gel was rinsed with *aqua dest.*. Subsequently, the color was developed by incubation in 100 ml of an aqueous solution of 2.5% (v/v) sodium carbonate and 0.01% (v/v) formaldehyde until the silver stained bands became visible. The reaction was stopped by incubation of

the stained gels in a 0.5 M EDTA solution for ten minutes. All reactions were performed at room temperature and with gently shaking on a table rocker.

3.9 Methods for the determination of protein concentration

3.9.1 The Lowry method modified by Peterson

Protein determination was performed according to the Lowry method modified by Peterson ((Lowry, et al., 1951; Peterson, 1977)) using the Sigma Protein Assay Kit No. P5656 (Bensadoun and Weinstein, 1976). The procedure was performed as described in the users manual with minor modifications. Samples were incubated with deoxycholate (0.125 mg/ml final concentration) for ten minutes at room temperature. Then, trichloracetic acid was added to a final concentration of 6% (w/v), vortexed and the mixture was incubated for 15 min at room temperature. To pellet the precipitated protein, the suspension was centrifuged for 15 min at 13,000 g (Heraeus Biofuge fresco, Heraeus Instruments GmbH, Berlin, Germany) at 15°C. The resulting supernatant was discarded. The sedimented protein precipitates were dissolved and rigorous vortexed in one milliliter SDS containing Lowry Reagent Solution and incubated for 20 min at room temperature. Subsequently, 0.5 ml of Folin & Ciocalteu's Phenol Reagent Working Solution were added, immediately and intensive vortexed and the color was allowed to develop for 45 min at room temperature. The absorbance was measured versus a blank sample at a wavelength of 750 nm (UV 2102 PC spectrometer, Shimadzu Europe GmbH, Duisburg, Germany). The measurements were completed within ten minutes. The protein content was determined by equal treated references of BSA standards with known concentrations.

3.9.2 The bicinchoninic acid (BCA) Method

An alternative method to rapidly determine the protein content of lipid and detergent containing samples, is the bicinchoninic acid (BCA) method in combination with SDS treatment. For the determination of protein concentration, the BCA Kit obtained from Pierce (Pierce, Rockford, IL, USA) was used, strictly

following the manual instructions. To minimize the influence of lipids on the protein determination reactions, aliquots of samples were mixed with SDS to a final concentration of 1% (w/v) SDS. In parallel, BSA standards with known concentrations were treated identically to generate a standard curve for protein quantification.

3.10 The lipid extraction procedure

Lipid extraction was performed according to Bligh and Dyer (Bligh and Dyer, 1959). An aliquot of membrane suspension was dissolved in *aqua dest.* to a final volume of 400 µl. Then, 500 µl chloroform and 1000 µl methanol were added. The suspension was rigorously vortexed for one minute. Subsequently, 500 µl chloroform, 500 µl methanol and a drop of 1 N HCl were added and the mixture was vortexed again for one minute. After centrifugation (10 min, 1,000 g, 4°C), the lower organic phase was collected in a Schott Duran™ glass tube (Hermann Kröpke GmbH, Berlin, Germany). The upper inorganic phase was extracted again by addition of one milliliter of chloroform, vortexed for one minute and centrifuged. The lower phase was collected and pooled with the first. If required, the re-extraction was repeated once more. The organic solvent was removed under a gentle stream of Nitrogen by using a thermostatically controlled heating block equipped with a multiple probe evaporator (Liebisch Thermochem-Metallblock-Thermostat/Multiplex-Ventil-Depot, Gebr.Liebisch GmbH & Co., Bielefeld, Germany).

3.11 Quantitation of phospholipids

The total phospholipid content of phospholipid containing samples was determined by measurement of the phospholipid phosphorus according to Rouser (Rouser, 1966). First, the lipids were extracted after the method of Bligh & Dyer (see 3.10). After evaporation of the organic solvent (see 3.10), 400 µl perchloric (72% (w/w)) acid was added and the samples were boiled for 1.5 h at 180°C in a thermostatically controlled heating block (Blockthermostat BT200, Kleinfeld Labortechnik, Hannover, Germany). After the solutions had cooled down, four

milliliters of Molybdate reagent (0.22% (w/v) ammonium molybdate, 0.25 M sulfuric acid) and 500 µl of 10% (w/v) ascorbic acid were added with mixing and incubated for ten minutes in a boiling water bath. The tubes were allowed to cool. The absorbance was read at 812 nm versus a blank sample. The phospholipid content of IIMV, proteoliposomes, liposomes and phospholipid containing suspensions were calculated from appropriately generated Na_2HPO_4 standard curves.

3.12 Detergent determination

Triton X-100 absorbance at 275 nm was used to check the removal of detergent during reconstitution as described by Hrafnsdottir and Menon (Hrafnsdottir and Menon, 2000). To this end, 150 µl of sample were mixed with 600 µl of methanol and 300 µl of chloroform and vortexed. The suspension was centrifuged (15,000 g, 15 min, 15°C) to remove the precipitated protein and the absorbance at 275 nm was measured. To determine the Triton X-100 concentration, identically treated detergent standards were assayed.

3.13 The measurement of the purity of isolated IIMV

To test the degree of contamination of the IIMV preparation with outer membrane fractions the activity of PLA_2, an outer membrane marker, was measured. To this end, five nanomol of head group labeled N-Rh-PE were diluted into one milliliter chloroform. Eleven microliter of this suspension were dried under a gentle stream of nitrogen, resuspended in five microliters of 50 mM Tris (pH 8), 20 mM calcium chloride, 0.2% Triton X-100. Subsequently, five microliters of the respective fraction were added and the incubation mixture was allowed to react for one hour at 37°C. After incubation, 22 µl of chloroform/methanol (1:1) were added, vortexed and centrifuged at 1,000 g for two minutes. Thin layer chromatography (TLC) of the lower phase was carried out with chloroform:methanol:water (65:25:4) on silica 60 plates. The spots were analyzed using a VD 40 Hitachi 3-Chip analyzing system (Desaga GmbH, Wiesloch Germany). PLA_2 activity was detected by spots corresponding to the

lyso derivative of N-Rh-PE. The analysis was performed with the standard accessory software (ProViDoc 3.04).

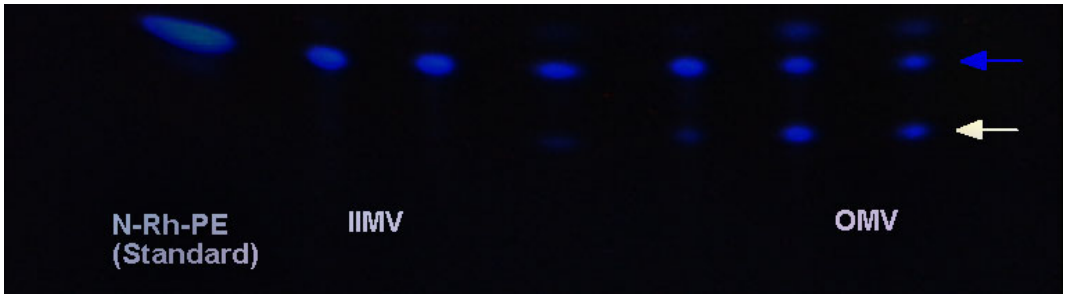


Figure 4: Representative experiment of detection of the PLA_2 activity in IIMV and outer membrane vesicles (OMV) derived from *E.coli*. The white arrow indicates the lyso derivatives of the PLA_2 activity. The blue arrow indicates the non-cleaved N-Rh-PE molecules by PLA_2.

In fractions of the outer membrane, a high degree of Phospholipase A2 activity was detected. The appearance of lyso N-Rh-PE (indicated with the white arrow in Figure 4) in the fraction of IIMV was below the detection level. Thus, IIMV are not or less contaminated with outer membrane fragments.

3.14 Kinetic analysis

The experimental data were fitted to a theoretical time course using a three-compartment model (Figure 5). This kinetic model describes transbilayer movement as well as the transfer of phospholipid analogues between the outer leaflet of the membrane vesicle and BSA (Marx, et al., 2000).

The outward and inward movements of phospholipid analogues are described by the rate constants k_{+1} and k_{-1}, respectively. The movement of the analogues from the IIMV to BSA is characterized by the rate constant k_{+2} (extraction of the analogues by BSA) and k_{-2} for the movement of analogues back from BSA to the vesicle membrane. Due to the excess of BSA used, the exchange process described by k_{-2} did not contribute to the kinetics and the values for this time constant were very small (typically 10^{-12} s^{-1}).

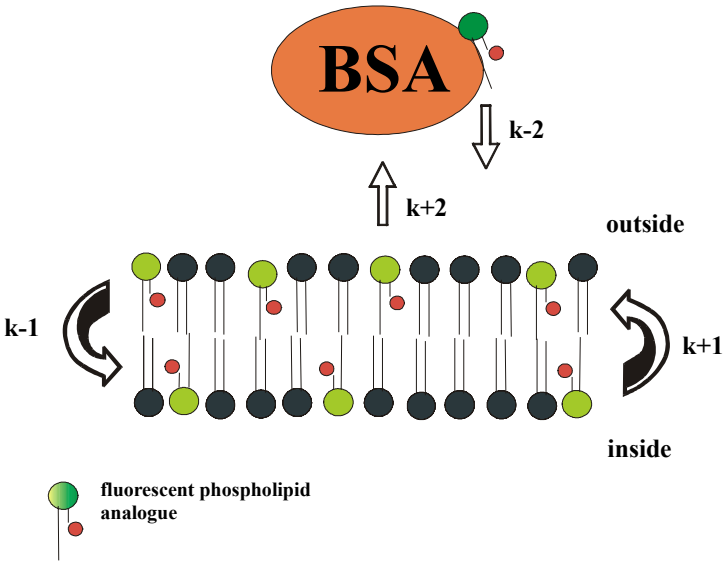


Figure 5: Model of the transbilayer movement of phospholipid (analogues) across membranes and extraction of fluorescent NBD-lipid analogues by BSA. The rate constants k_{-1} and k_{+1} indicate the inward and outward movement of phospholipid analogues across the membrane, respectively. The extraction of fluorescent analogues is described by k_{+2} and the movement from BSA to the membrane is indicated by k_{-2}.

$[PL_o]$ and $[PL_i]$ are the concentrations of analogue in the outer and inner leaflet of the IIMV. At the time of BSA addition (t = 0s), the transmembrane distribution is at steady state, i.e.,

$$\frac{[PL_o]_{t=0}}{[PL_i]_{t=0}} = \frac{k_{+1}}{k_{-1}} \tag{1}$$

The concentration of analogue transferred to BSA $[PL_{tr}]$ is taken to be zero at the time of addition of BSA.

The model is represented by the following system of differential equations:

$$\frac{d[PL_i]}{dt} = -k_{+1}[PL_i] + k_{-1}[PL_o] \tag{2}$$

$$\frac{d[PL_o]}{dt} = k_{+1}[PL_i] - (k_{-1} + k_{+2})[PL_o] + k_{-2}[PL_{tr}] \tag{3}$$

$$\frac{d[PL_{tr}]}{dt} = k_{+2}[PL_o] - k_{-2}[PL_{tr}] \tag{4}$$

$$[PL_{tr}] = C - [PL_i] - [PL_o] \tag{5}$$

$$C = [PL_i]_{t=0} + [PL_o]_{t=0} + [PL_{tr}]_{t=0} \tag{6}$$

For further details see (Marx, et al., 2000). Fitting was performed by least-square minimization.

4 Results

One goal in this study was to characterize the transbilayer distribution and transbilayer movement of fluorescence labeled phospholipid analogues in the inner membrane of *E.coli*. The phospholipid flip-flop in native and reconstituted *E.coli* inner membranes was analyzed using the BSA back-exchange stopped-flow assay developed by Marx *et al.* (Marx, et al., 2000) in combination with the tool of reconstitution of membrane proteins (Hrafnsdottir and Menon, 2000; Hrafnsdottir, et al., 1997; Menon, et al., 2000). Earlier studies on the translocation process of phospholipids could not demonstrate a clear requirement of proteins and/or lacked a sufficient time resolution (Hrafnsdottir and Menon, 2000; Hrafnsdottir, et al., 1997; Huijbregts, et al., 1996; Huijbregts, et al., 1998). Therefore, the transmembrane distribution and movement of fluorescent short-chain phospholipid analogues in IIMV were characterized by using the highly time resolving stopped-flow technique (chapter 4.2 and 4.3). To extend the progress in characterization of the putative flippase, triton extracts (TE) of the inner membrane of *E.coli* were reconstituted (4.3). The transbilayer movement of different fluorescent phospholipid analogues in proteoliposomes derived from IIMV of *E.coli* was characterized using the BSA back-exchange and the dithionite assay (see chapter 4.3, 4.4 and 4.5). Furthermore, strong evidence were found for an involvement of protein in this translocation process as shown by protease treatment and reconstitution experiments (4.6). Moreover, the flippase activity could be recovered from fractions after chromatographic separation of solubilized IIMV (4.7).

4.1 Incorporation of fluorescent phospholipid analogues into IIMV

In aqueous solution, the NBD-labeled phospholipid analogues are mainly organized in micelles where their fluorescence is self-quenched. Upon adding acceptor membranes, such as IIMV or pure phospholipid vesicles, the fluorescence increases, since the fluorescent labeled phospholipid molecules spontaneously incorporate into IIMV and thus, micelles are dissolved. Incorporation of fluorescent lipids into membranes can therefore be directly monitored by fluorescence increase.

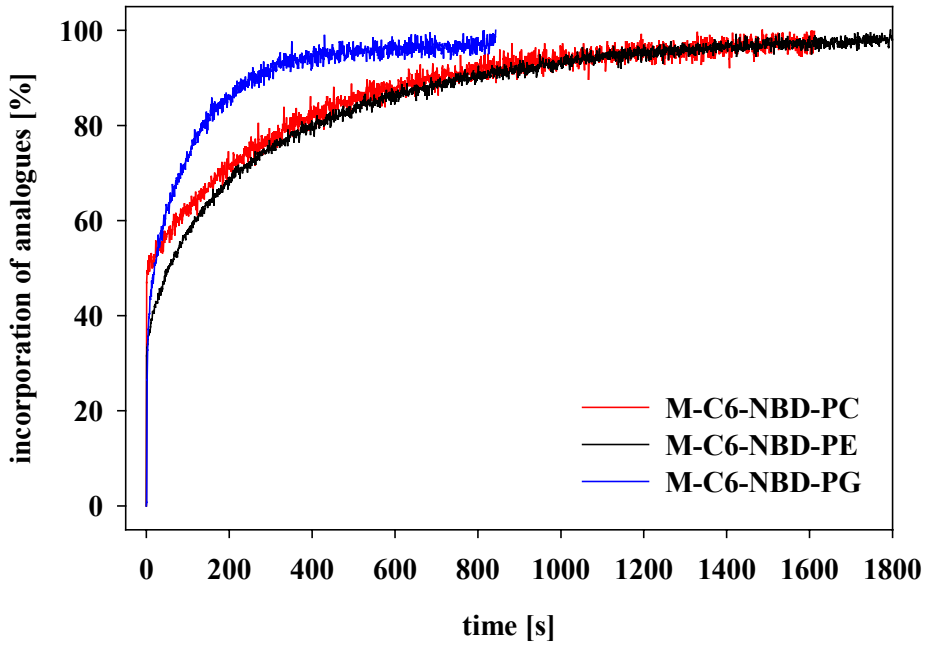


Figure 6: Kinetics of incorporation of NBD-labeled phospholipid analogues into IIMV membranes. Two molpercent of M-C6-NBD-PE, M-C6-NBD-PC or M-C6-NBD-PG in 10mM HPS, with respect to the total lipid content of the IIMV, was added to IIMV (12.5µM final phospholipid concentration), and the kinetics of membrane incorporation of the analogues were monitored. At time point zero, the IIMV were added to the label suspension. The value of 100% corresponds to incorporation of all fluorescent labeled analogues. All experiments were performed at room temperature. Due to the low time resolution, the initial fluorescence increase upon mixing of IIMV with lipid analogues could not be adequately resolved.

To analyze the incorporation of fluorescent lipid analogues into inner membrane vesicle from *E.coli*, an aliquot of IIMV was mixed with 10 mM HPS containing two molpercent of M-C6-NBD-PC, -PE or M-C6-NBD-PG in a quartz cuvette and the resulting increase of fluorescence intensity was monitored using a fluorescence spectrometer (see 3.4).

As evident from Figure 6, an initial rapid phase of intercalation of analogues into IIMV was observed. After ten minutes, about 90% of M-C6-NBD-PE and -PC were incorporated. However, the final plateau was reached within 30 min. Essentially all of the PE and PC analogues were intercalated into the IIMV. Moreover, the kinetics of incorporation of the fluorescent analogues of PE and PC were very similar. In contrast to these phospholipids analogues, M-C6-NBD-PG was much faster incorporated into IIMV. A stable plateau of fluorescence intensity was reached within ten minutes i.e., all of the NBD-labeled PG was incorporated within this time.

To verify, whether all analogues were incorporated into IIMV after a final plateau was attained, an extra aliquot of vesicles was added. After addition of an extra aliquot of vesicles no further fluorescence increase was observed (data not shown), indicating that all of the analogues were incorporated into the IIMV.

4.2 Transbilayer movement of fluorescent phospholipid analogues across IIMV membranes

For the measurement of the transbilayer movement of M-C6-NBD-PE, M-C6-NBD-PC and M-C6-NBD-PG across the IIMV membranes, stopped-flow method was used taking advantage of the fact that short-chain lipid analogues can be extracted from the membrane by BSA (BSA back-exchange), and that the quantum yield of analogues bound to BSA is different from that of membrane incorporated analogues.

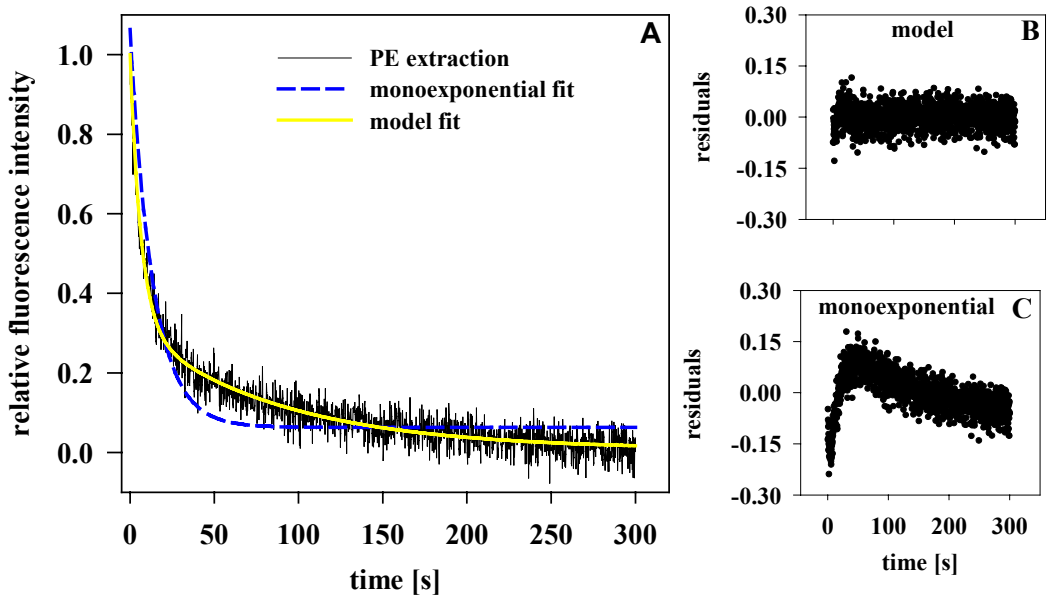


Figure 7: Kinetics of extraction of M-C6-NBD-PE from IIMV by BSA. An aliquot (25 µl) of IIMV (2.89 mg protein/µmol phospholipid) were incubated with two milliliters of a buffered suspension of M-C6-NBD-PE (two molpercent of the lipid content) for 30 min at room temperature. Subsequently, the labeled IIMV were rapidly mixed with an equal volume of 4% (w/v) BSA in a stopped-flow accessory. The fluorescence decay was recorded with a time resolution of 0.2 s. The kinetics were normalized as follows: The initial fluorescence intensity (before BSA extraction) was set to one, the intensity after 300 s to zero. The curve represents the average of five measurements (A). The solid yellow line represents the fit obtained by fitting the data to the three-compartment model. The dashed blue line was obtained by fitting the data to a monoexponential function. The residuals of the model fit (B) according to the three-compartment model (3.14) and the monoexponential fit (C) are depicted.

After labeling of IIMV with max. two molpercent of fluorescent analogues for 30 min at room temperature, vesicles were rapidly mixed with 2% (w/v) BSA (final concentration) in 10 mM HPS by stopped-flow. The time dependent decrease of fluorescence intensity resulting from back-exchange of analogues by BSA was monitored.

Table 1: Half-times of transbilayer movement of NBD-labeled phospholipids across the membrane of IIMV and of extraction of analogues from the outer leaflet.

analogue	outward movement [s]	inward movement [s]	extraction [s]	$[PL_i]_{t=0}$ [%]
M-C6-NBD-PE	58	160	4.6	26.6
	48	210	2.5	18.6
M-C6-NBD-PC	17	100	1.1	14.7
	63	242	2.7	20.7
M-C6-NBD-PG	64	55	3.3	53.8
	65	97	4.4	40.1
P-C6-NBD-PS	52	136	15.5	27.7
	65	196	14.9	24.9

$[PLi]_{t=0}$ refers to the amount of analogues at time point of BSA addition

As shown in Figure 7 (displayed for M-C6-NBD-PE), the fluorescence emission intensity was found to decay in two distinct phases. After 300 s no change of fluorescence was observed, suggesting that all phospholipid analogues were extracted and bound to BSA (see below). Therefore, the kinetics were normalized as follows: the initial fluorescence intensity (before BSA extraction) was set to one, the intensity after 300 s to zero. The fast initial decrease of fluorescence intensity reflects the extraction of phospholipid analogues localized in the outer leaflet of the vesicles. Furthermore, the second slower phase is caused by extraction of M-C6-NBD-PE translocated from the lumenal leaflet to the outer leaflet. To determine the characteristic half-times of the two phases, the data were fitted as described in 3.14 to the three-compartment model (Figure 7A, yellow line). Additionally, a monoexponential fit was performed (Figure 7A, blue dashed line) to compare the parameter based on the three-compartment model with a simple monoexponential process. The residuals - the differences between measured and fitted values - clearly showed that a monoexponential function did not fit the recorded data (Figure 7C). However, the data could be well fitted by a model process yielding four rate constants (Figure 7A, and Figure 7B). Based on the three-compartment model (see 3.14), the half-times of flip-flop, extraction of analogues as well as their initial transbilayer distribution were estimated (Table 1). The transbilayer dynamics and distribution of M-C6-NBD-PE and M-C6-NBD-PC were found to be very similar (Table 1).

The same results were observed for a fluorescent analogue of PS, which contains an elongated fatty acid on the sn-1 position. Despite of the palmitic acid (16 carbon atoms) instead of myristic acid, the slightly enhanced hydrophobicity of this analogue had no influence of the characteristic transbilayer movement of P-C6-NBD-PS. Moreover, the half-times of flip-flop and the transbilayer distribution of P-C6-NBD-PS were almost identical to those found for M-C6-NBD-PE (Table 1).

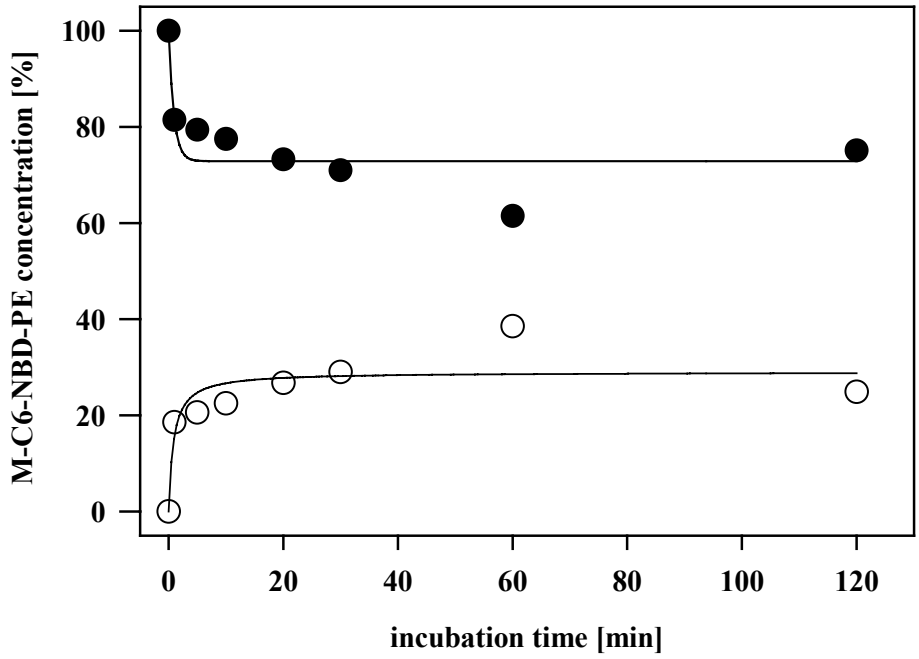


Figure 8: Time dependence of extraction of M-C6-NBD-PE at room temperature. An aliquot of IIMV was mixed with buffer containing two molpercent of M-C6-NBD-PE of the total lipid content and the mix was immediately transferred to the stopped-flow device. Subsequently, the IIMV/label suspension was rapidly mixed with an equal volume of 4% (w/v) BSA in a stopped-flow accessory and the fluorescence decay was recorded with a time resolution of 0.5 s at the indicated time points. The kinetics were normalized as described before, and the data were fitted to the three-compartment model. The transbilayer distributions were elucidated from the fits and are displayed as function of incubation time of the analogues with IIMV. The open symbols refer to the concentration of analogues in the inner leaflet, the closed symbols to the concentration of analogues in the outer leaflet of the IIMV. The solid lines display simple one-phase regressions.

While data in Table 1 refer to measurements on IIMV samples labeled for 30 min with NBD-lipids, experiments were carried out to test the efficiency of labeling and the influence of long term incubation of the IIMV with fluorescent lipid analogues. An aliquot of IIMV was mixed with buffer containing two molpercent of the appropriate fluorescent lipid analogue with respect to the total lipid concentration of IIMV. This mixture was rapidly transferred to the stopped-

flow device. This procedure took less than 90 s. Subsequently, the stopped-flow BSA back-exchange assay was performed immediately as described above. Briefly, at time points 0, 5 min, 10 min, 15 min, 20 min, 30 min, 60 min and 90 min the IIMV-NBD-lipid suspension and 4% (w/v) BSA were mixed in the stopped-flow chamber and extraction kinetics were monitored. The kinetics were fitted according to the three-compartment model (see 3.14), and the resulting transbilayer distributions of analogues were displayed *vs.* incubation time of analogues (Figure 8 – shown for M-C6-NBD-PE). Up to 15 min after addition of analogues to IIMV, the distribution of the fluorescent lipids and the half-times of transbilayer movement were dependent on the incubation time. After 20 min, no changes in distribution and movement were found, when performing stopped-flow measurements on IIMV preparations labeled up to 90 min (Figure 8). This indicates that after 20 min of labeling, the analogues were equilibrated between the two leaflets of the bilayer, consistent with a rapid flip-flop of the phospholipid analogues.

The kinetics displayed in Figure 8 were fitted to a simple first order function. The resulting half-times were $t^{i}_{1/2}$ ~1 min and $t^{o}_{1/2}$ ~1.95 min for the inner and outer leaflet, respectively. These data are in agreement with the half-times calculated from the stopped-flow BSA back-exchange assay stated in Table 1.

To verify whether all analogues were extracted by BSA, an aliquot of IIMV was labeled with two molpercent of M-C6-NBD-PE or M-C6-NBD-PC for 30 min, then the vesicles were incubated with 2% (w/v) BSA for 300 s at room temperature and the resulting NBD-fluorescence intensity was measured. The fluorescence intensity was the same as that found, when an equal amount of analogues in aqueous suspension was incubated with 2% (w/v) BSA (Figure 9B, C - only shown for M-C6-NBD-PE) and about 55% of that seen, when the analogues were all membrane integrated (compare Figure 9B with A).

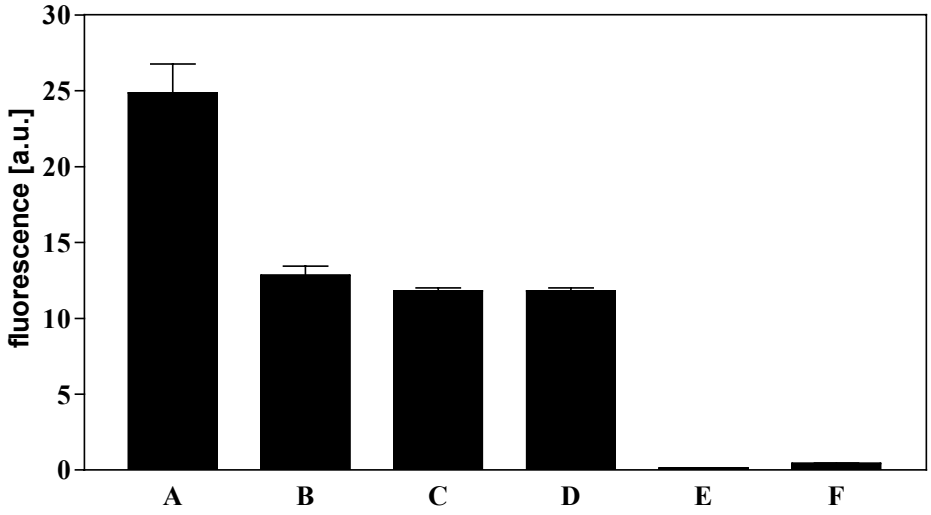


Figure 9: Complete extraction of M-C6-NBD-PE from IIMV by BSA. (A) IIMV (60 µM final phospholipid concentration) were labeled with two mol% M-C6-NBD-PE for 30 min, (B) subsequently, the analogues were extracted by 2% (w/v) BSA in 10 mM HPS for 300 s. Longer incubation (>300 s) did not change the fluorescence intensity. (C) BSA (2% (w/v))-bound M-C6-NBD-PE (two mol% of total phospholipid content of IIMV) in the absence of IIMV. (D) An aliquot of IIMV was added to BSA-bound M-C6-NBD-PE (two mol% of total phospholipid content of IIMV). (E) IIMV (equal to the amount used in (A) and (D)) in two ml 10 mM HPS. (F) 2% (w/v) BSA in two milliliters 10 mM HPS. The error bars indicate the standard deviation of two experiments. The experiments were performed at room temperature.

Our data clearly show that all PE or PC analogues were completely extracted by BSA after 300 s. When an aliquot of IIMV was added to the sample containing the BSA-bound analogues no changes in fluorescence occurred due to the presence of unlabeled IIMV (Figure 9D). This is consistent with the assumption that k_{-2} (transfer of analogues from BSA to membranes) contributes very little to our analysis (Figure 5).

4.3 Transbilayer movement of fluorescent phospholipid analogues across the membrane of reconstituted proteoliposomes derived from IIMV

To analyze the relevance of proteins for phospholipid flip-flop, proteoliposomes containing detergent solubilized protein extract from inner membrane vesicles and two molpercent M-C6-NBD-PE were reconstituted as described in chapter 3.3. By this procedure ~50-70% of the protein and ~60-85% of the phospholipids from the reconstitution mixture were recovered in the resulting proteoliposomes. Essentially all of the initial Triton X-100 (>99.9%) was removed (data not shown).

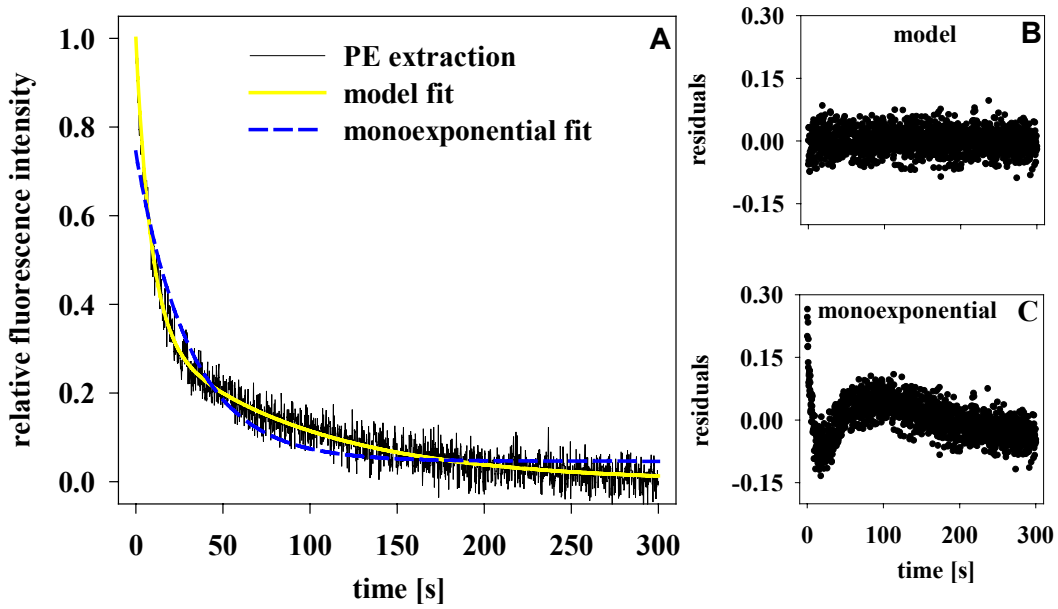


Figure 10: Stopped-flow kinetics of extraction of M-C6-NBD-PE from reconstituted IIMV by BSA. IIMV-derived proteoliposomes were labeled with two molpercent of the fluorescent phospholipid analogues during the reconstitution as described in 3.3. In brief, equal volumes of proteoliposomes and 4% (w/v) BSA were mixed in the stopped-flow apparatus and the fluorescence decrease was recorded at room temperature (A). The curve represents the average of five separate kinetic traces. Kinetics were corrected for scattering. The solid yellow line represents the fit of the experimental data to the three-compartment model shown Figure **5**. The dashed blue line was obtained by a monoexponential fit of the data. The residuals for the fit of the data to the three-compartment model (B) and of a monoexponential fit (C) of the extraction kinetics are displayed.

The proteoliposomes were rapidly mixed with BSA (2% (w/v) final) by stopped-flow, and the time-dependent decrease of fluorescence intensity caused by BSA back-exchange of the analogues was monitored.

Figure 10A displays the kinetics of extraction of M-C6-NBD-PE from IIMV derived reconstituted proteoliposomes. As with IIMV, a similar bi-phasic decline of the fluorescence intensity on adding BSA to the M-C6-NBD-PE-labeled proteoliposomes was found (Figure 10A). Again, the BSA back extraction of M-C6-NBD-PE could not be fitted by a monoexponential function (blue dashed line in Figure 10A and panel C). Therefore, the kinetics were analyzed by the three-compartment model (yellow solid line in Figure 10A and panel B). As evident from Figure 10B, this model fitted the back extraction kinetics excellently. From fitting of the experimental data to the model, it was determined that transbilayer movement of NBD-phospholipids in proteoliposomes was very similar to that seen in IIMV. The respective rate constants were in the same order as those found for IIMV (data not shown). From the analysis of five independent experiments (n=5) a half-time for the outward movement of M-C6-NBD-PE of t_{k+1}= 29 s, for the inward movement t_{k-1}= 70 s and a half-time of extraction t_{k+2}= 5 s was estimated (Table 2). The relative amount of analogues residing at the lumenal side at time point zero was $[PL_i]_{t=0}$= 29%. The rate constant of the backward movement of M-C6-NBD-PE from BSA to the proteoliposomes given by the fit was very small (k_{-2}= $10^{-12}s^{-1}$) and is therefore negligible.

Table 2: Half-times of transbilayer movement of NBD-labeled phospholipids across the membrane of reconstituted IIMV-derived proteoliposomes and extraction of analogues from the outer leaflet

analogue	outward movement [s]	inward movement [s]	extraction [s]	$[PLi]_{t=0}$ [%]
M-C6-NBD-PE (n=5)	28.7 ± 2.2	69.5 ± 3.7	5.16 ± 0.73	29.2
M-C6-NBD-PG (n=3)	119.3 ± 7.0	69.7 ± 2.8	4.2 ± 0.07	63.1

$[PLi]_{t=0}$ refers to the amount of analogues at time point of BSA addition

In Figure 11 a representative experiment for the extraction of M-C6-NBD-PE from pure lipid vesicles (liposomes) made from ePC is shown. The liposomes (black line in Figure 11 A) were labeled with M-C6-NBD-PE during the reconstitution and subsequently, incubated with 4% (w/v) buffered BSA in a stopped-flow device. The fluorescence intensity decayed with a half-time of about eight seconds. The fluorescence intensity plateau leveled off at ~80% of the initial

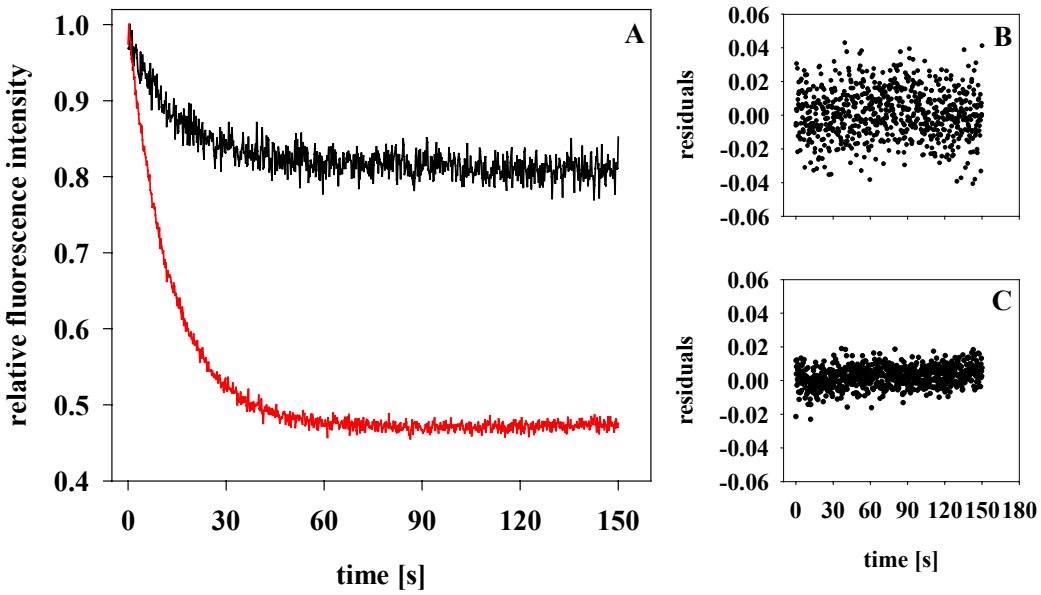


Figure 11: Extraction kinetics of pure ePC-vesicles. Control vesicles were prepared in parallel to proteoliposomes but without bacterial protein as described in 3.3. (A) Pre-labeled ePC-vesicles (black line) were prepared by adding two molpercent M-C6-NBD-PE during the reconstitution procedure, whereas post-labeled ePC-vesicles (red line) were incubated with two molpercent of the same analogue prior the fluorescence measurement for 30 min. The stopped-flow back-exchange assay was performed as described for proteoliposomes (see Text and Figure **10**). In panels B and C the residuals resulting from monoexponential fits from pre-labeled and post-labeled ePC-vesicles, respectively, are shown.

fluorescence intensity. This is consistent with the extraction of ~50% of analogues from IIMV (compare to Figure 9). As evident from the residuals displayed in Figure 11B, the extraction kinetics of protein-free liposomes (Figure 11 A) could be fitted to a monoexponential function. The residuals yielded from this fit showed no time dependence.

For comparison, the BSA back-extraction kinetics of pure lipid liposomes labeled with the same amount of M-C6-NBD-PE after reconstitution (red line) is shown in Figure 11. The post-labeled liposomes exhibited a similar monoexponential fluorescence intensity decline, resulting in a final intensity plateau of ~50% of the initial fluorescence emission intensity. This value corresponds to the complete extraction of the fluorescence analogues from the vesicles. Furthermore, it can be concluded that no flip-flop of phospholipid analogues occurred across the vesicle membrane in the absence of bacterial proteins. These results are consistent with the interpretation that the initial phase corresponds to extraction of the analogues from the outer leaflet, while the second slower phase corresponds to the transbilayer movement.

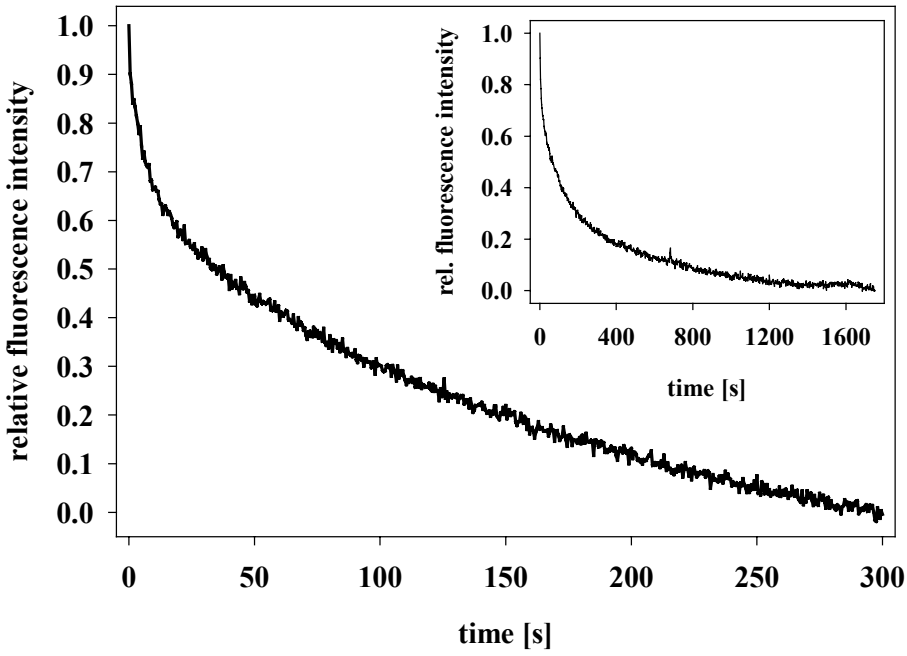


Figure 12: Stopped-flow-kinetics of extraction of M-C6-NBD-PG from reconstituted IIMV by BSA. IIMV-derived proteoliposomes were labeled with two molpercent of the fluorescent phospholipid analogues during the reconstitution as described in 3.3. Equal volumes of proteoliposomes and 4% (w/v) BSA were mixed in the stopped-flow apparatus, and the fluorescence decrease was recorded at room temperature. A typical fluorescence kinetics of extraction of the fluorescent PG analogue is shown. The fluorescence intensity did not reach a stable plateau after 300 s of BSA incubation. In the inset, representative extraction kinetics of M-C6-NBD-PG with an elongated incubation time to 30 min is shown. Essentially all PG analogues were extracted by BSA after 30 min. The experiments were carried out at room temperature.

In order to characterize the membrane translocation of an analogue of the second major phospholipid in the inner membrane of *E.coli* - PG - identical experiments as for M-C6-NBD-PE were performed as described above for M-C6-NBD-PE.

As for the fluorescent PE analogue, the fluorescence decay was essentially a bi-phasic process. As already concluded by the above given arguments, the initial decrease of fluorescence intensity reflects the extraction of phospholipid analogues localized in the outer leaflet of the vesicles. The second slower phase was caused by extraction of M-C6-NBD-PG translocated from the lumenal leaflet to the outer leaflet. The half-times of transbilayer movement of M-C6-NBD-PG across the membrane of proteoliposomes were 119 s and 70 s for the outward and inward movement, respectively (Table 2).

The rate of outward movement of the fluorescent PG analogue is about four times slower than that of PE analogues. As deduced from the model, the lumenal concentration of M-C6-NBD-PG at time point zero was about two times higher

(63.1%, see Table 2) as for PE analogues. The extraction of M-C6-NBD-PG was not finished after 300 s (Figure 12). However, after 300 s more than 90% of the PG analogues were extracted by BSA. As evident from Figure 12A only after 30 min essentially all of the PG analogues were extracted. This is consistent with the findings for the initial distribution of the PG analogue.

4.4 Effect of proteins on the transbilayer movement of phospholipids

4.4.1 Extraction of M-C6-NBD-PE from IIMV membranes

To analyze the mechanism of phospholipid flip-flop further, series of proteoliposomes from IIMV containing different amounts of detergent solubilized protein extract and two molpercent M-C6-NBD-PE were reconstituted as described in chapter 3.3. The reconstituted proteoliposomes were rapidly mixed with a BSA solution (4% (w/v) in 10 mM HPS) by stopped-flow, and the time dependent decrease of fluorescence caused by back-exchange of analogues by BSA was monitored.

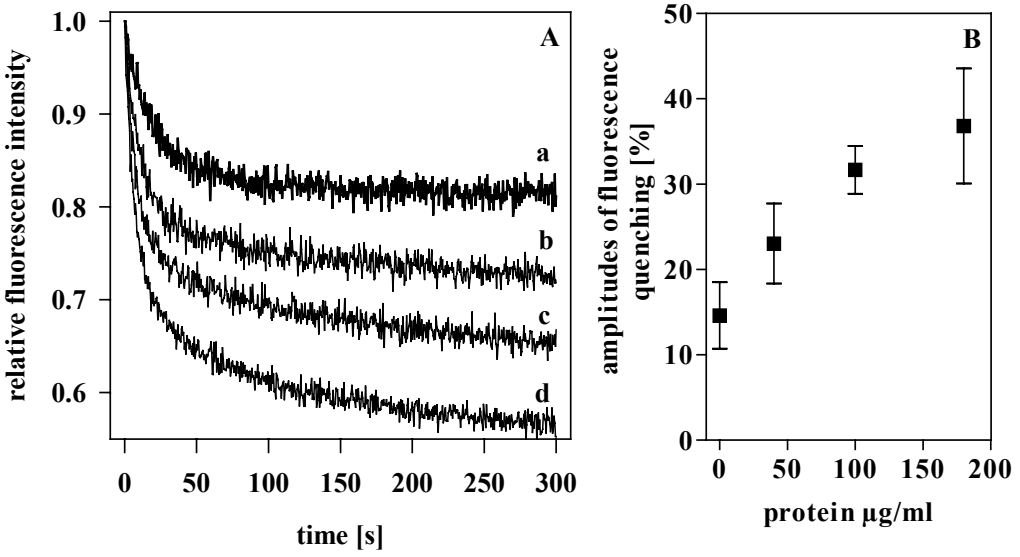


Figure 13: Effect of protein concentration on flip-flop and extraction of M-C6-NBD-PE. (A) The proteoliposomes were prepared with 4.5 mM ePC, increasing amounts of the TE and labeled with M-C6-NBD-PE during the reconstitution procedure. Subsequently, the labeled proteoliposomes made from IIMV were analyzed by stopped-flow assay as described before. The kinetics represent the mean of at least six records corresponding to pure ePC-liposomes (trace a), and to proteoliposomes with 40 µg protein/ml (trace b), 100 µg protein/ml (trace c) and 180 µg protein/ml (trace d). (B) The final amplitudes of fluorescence decrease (see (A)) of two independent experiments with increasing amounts of proteins are shown. The error bars depict the standard deviation. The letters a-d next to each data point correspond to the traces (for one of the two experiments) in panel A.

At high protein content (180 µg/ml, Figure 13A trace d), it was observed that the fluorescence decreased finally to about 55% of the initial value. Based on quantum yields, this indicates that most of the analogues (≥90%) were extracted from the proteoliposomes to BSA. At lower protein content, removal of analogues

was not complete, possibly indicating that a fraction of the proteoliposomes lacked the putative flippase protein (Figure 13 traces b and c).

For vesicles reconstituted in the absence of protein extract but in the presence of Triton X-100, only the rapid initial phase of fluorescence decline but no further (slow) decrease (Figure 13A) was observed. The fluorescence in the liposomes samples reached a final plateau between 80 and 85%, consistent with the removal of about 50% of the analogues by BSA (see also Figure 9).

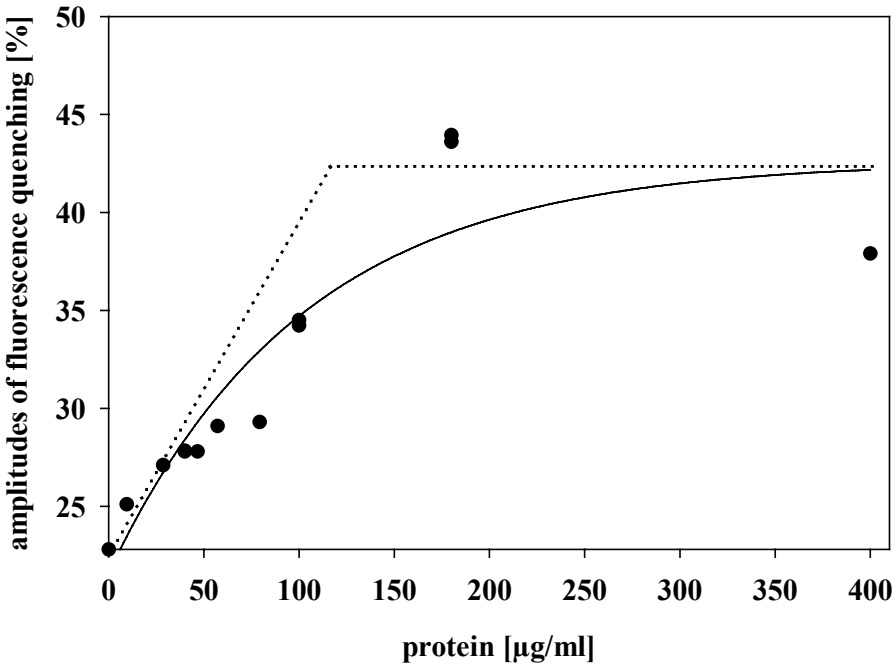


Figure 14: Protein dependence of transmembrane movement and extraction of M-C6-NBD-PE. Proteoliposomes containing fluorescent phospholipid analogues and increasing amounts of TE were assayed by stopped-flow as described in the text. The percentage of fluorescence decrease of six independent experiments is depicted.

The amplitude of the extraction of M-C6-NBD-PE was proportional to the protein/phospholipid ratio in the range 0-100 µg/µmol. As evident from Figure 13B the amplitude of fluorescence reduction leveled off at a plateau, possibly indicating that at a protein/phospholipid ratio of ~100 µg/µmol almost every vesicle was equipped with at least one flippase and therefore, no further substantial increase in fluorescence intensity was observed.

In Figure 14 a composite of data obtained from a number of experiments using IIMV derived proteoliposomes reconstituted with different protein concentration is summarized. The inflection point of the dose response plot displayed in Figure 14 lays at a similar protein to phospholipid ratio as that

depicted in Figure 13B. This indicates that at this concentration (100 µg/ml) of proteins the majority of vesicles are equipped with proteins, which facilitate the rapid transbilayer movement of phospholipid analogues. Taken together, these data strongly indicate that proteins are involved in the rapid transbilayer movement of phospholipids across proteoliposome bilayer.

4.4.2 Reduction of M-C6-NBD-PE in IIMV-derived membranes by dithionite

To verify the results of the BSA back-exchange assay, an alternative assay was established to analyze the transbilayer movement of M-C6-NBD-PE across IIMV derived proteoliposomes. In the past, the dithionite assay has been successfully used for investigations of transmembrane movement and distribution of fluorescent lipid analogues (McIntyre and Sleight, 1991).

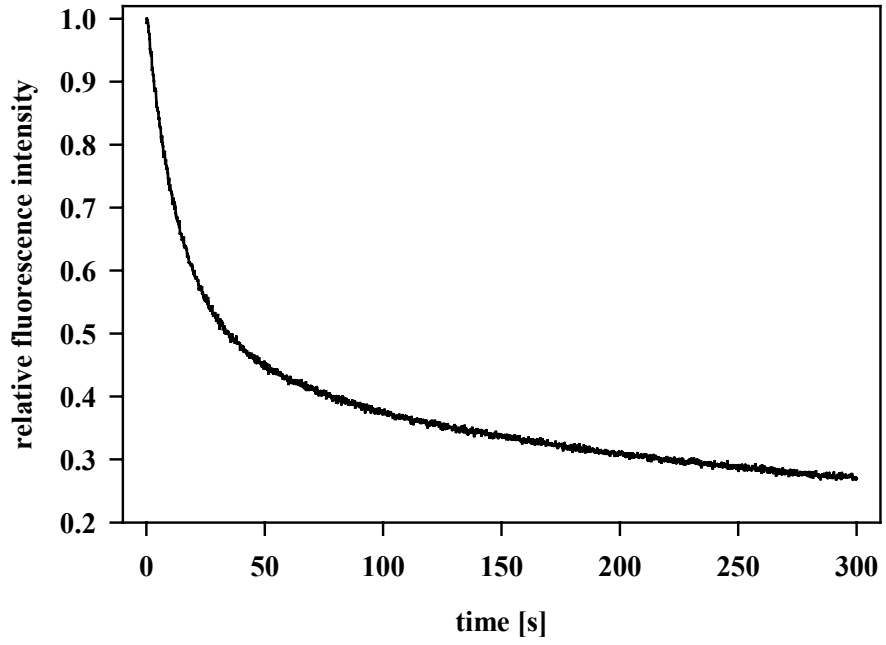


Figure 15: Stopped-flow kinetics of fluorescence quenching of M-C6-NBD-PE from reconstituted IIMV by dithionite. IIMV-derived proteoliposomes were labeled with one molpercent of the fluorescent phospholipid analogues during the reconstitution as described in 3.3. Equal volumes of proteoliposomes and 20 mM freshly prepared dithionite were mixed in the stopped-flow apparatus, and the fluorescence decrease was recorded at room temperature. A typical fluorescence kinetics of fluorescence reduction of the fluorescent PE analogue is shown. The protein content of the reconstituted proteoliposomes was 150 µg/ml.

Dithionite quenches the NBD fluorescence irreversibly by a chemical reaction (see 3.6 and Figure 3; Figure 15). Similar to the BSA back-extraction assay, when adding dithionite to a fluorescent labeled vesicle suspension, only the

fluorescent molecules residing on the outer leaflet will be accessible by dithionite. Subsequently, only on these molecules the fluorescence will be quenched.

To analyze the transbilayer movement of M-C6-NBD-PE in dependence of the protein content, series of proteoliposomes with different amounts of bacterial proteins were created, as described in chapter 4.4.1 (see also 3.3). The proteoliposomes were rapidly mixed with a freshly prepared dithionite solution (20 mM) by stopped-flow, and the decrease of fluorescence was monitored for 300s. In Figure 15 an example of a typical fluorescence reduction kinetics by dithionite is shown. As evident from Figure 15, the fluorescence intensity decays below 50% of the initial fluorescence intensity. Therefore, fluorescent PE analogues must have moved from the exoplasmic leaflet to the cytosolic leaflet and became accessible to dithionite (see discussion). Hence, the amplitudes of the dithionite fluorescence kinetics are useful for analyzing the ability of protein-mediated phospholipid flip-flop. The resulting amplitudes of fluorescence intensity were determined and are summarized in Figure 16.

Similar to the BSA back-extraction assay, the amplitudes of the final fluorescence intensity decreased with increasing amounts of bacterial protein reconstituted into proteoliposomes. Analyzing liposomes, i.e., pure lipid vesicles prepared in the presence of detergent but without proteins, the fluorescence intensity decayed to ~50% of the initial value, reflecting that only the outer leaflet populating fluorescent phospholipid analogues were reduced by dithionite (Figure 16), and no flip-flop occurred. With increasing quantity of proteins in the proteoliposomes the amplitudes of fluorescence decreased in the range 0-80 µg/ml protein. No consistent decrease in amplitude was observed, when proteoliposomes were prepared at a protein content greater than ~80 µg/ml.

The kinetic measurements, which form the basis of the data displayed in Figure 16, were analyzed by the three-compartment model (3.14). A precondition of this analysis is the impermeability of the reducing reagent dithionite. Moreover, the original three-compartment model was modified with respect to the absence of the fourth rate constant k_{-2} (movement of the analogue from BSA to the vesicle).

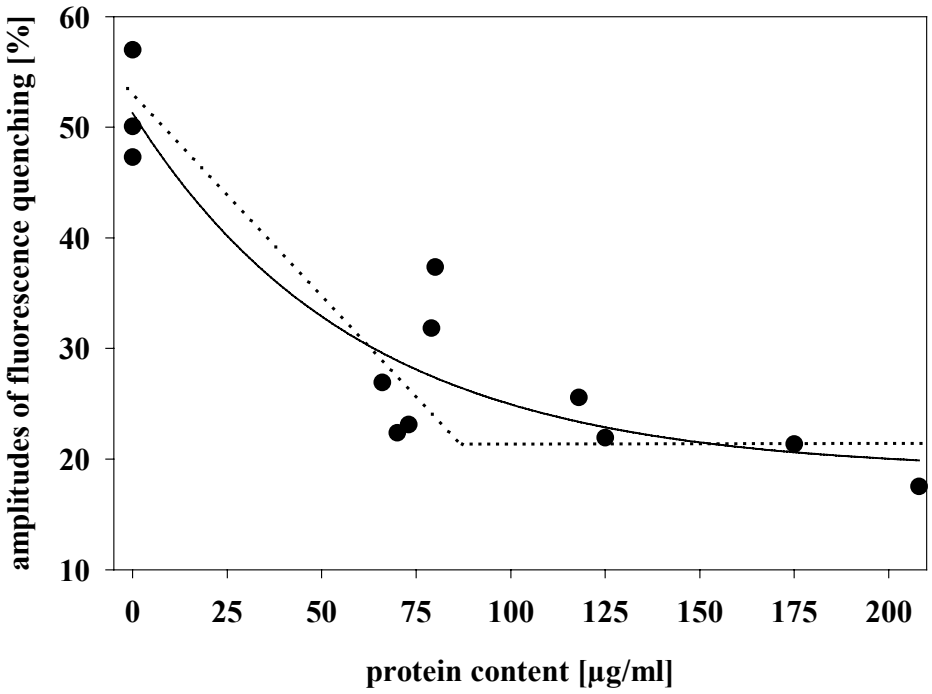


Figure 16: Protein dependence of transmembrane transport and extraction of M-C6-NBD-PE by the dithionite assay. Proteoliposomes containing one molpercent M-C6-NBD-PE and increasing amounts of TE were assayed by stopped-flow dithionite assay as described in 3.6. Each data point represents the mean of five dithionite kinetics. The percentage of fluorescence decrease of three independent experiments is summarized. The dotted line indicates the linear range and the plateau of protein content-dependent fluorescence intensity amplitudes.

Subsequently, the dithionite kinetics were fitted with three parameters: k_{-1}, k_{+1}, and k_{+2}; describing the outward movement, inward movement and fluorescence reduction by dithionite, respectively.

The results are presented in Table 3. The half-times of flip-flop of M-C6-NBD-PE were very similar to those found with the BSA back-extraction assay (Table 2). The half-times of outward movement were faster than for the inward movement of the analogue, similarly to half-times estimated from the BSA back-exchange assay. Interestingly, half-times determined from the dithionite assay were slower than those found by the BSA back-extraction assay. One possible explanation for this is that the proteoliposomes were slightly leaky for dithionite. Thus, the estimation of the rate constants for the transbilayer movement led to a small overestimation of the corresponding half-times.

Table 3: Half-times of transbilayer movement of M-C6-NBD-PE across *E.coli*-derived proteoliposomes determined by the dithionite assay. Three independent experiments (n=3) were analyzed by the modified three-compartment model.

protein content [µg/ml]	outward movement [s]	inward movement [s]	dithionite reduction [s]	fluorescence amplitude [%]
76	47.9 ± 6	135 ± 20.1	4.18 ± 0.55	68.0
87	40.6 ± 2.7	149.6 ± 18.9	5.96 ± 0.40	76.3
169	36.3 ± 6.1	156 ± 49.8	5.76 ± 0.56	79.7

However, the findings with the alternative dithionite stopped-flow assay further support the hypothesis that the transmembrane movement and the resulting distribution of the fluorescent PE analogue is fast and mediated by bacterial proteins.

4.5 Effect of the chain length of fluorescent phospholipid analogues on the transbilayer movement across IIMV-derived membranes

So far, short-chain fluorescent phospholipid analogues were used to study the transbilayer movement of phospholipids across IIMV membranes, taking advantage of the rapid and quantitative extractability of such analogues by BSA. Although the phospholipid analogues used, have structural features of endogenous phospholipids, it has to be taken into account, that these analogues possibly do not adequately mimic the endogenous phospholipids. It is conceivable that the particular structure of the fluorescent analogue may lead to different results than those obtained with natural phospholipids. To address whether long-chain or head-group labeled phospholipid analogues behave similarly to the so-far used short-chain fluorescent phospholipids, in terms of transbilayer movement and initial distribution between the two leaflets, experiments were carried out with reconstituted (proteo)liposomes containing the long-chain fluorescent analogue palmitoyl-dodecan-NBD-PE (P-C12-NBD-PE) or the head-group labeled N-NBD-dipalmitoyl- PE (N-DP-NBD-PE). Since BSA is not able to extract both long-chain phospholipid analogues efficiently and fast enough to get adequately rate constants for model analysis due to strong hydrophobicity of the longer fatty acids, a combined dithionite stopped-flow assay was established. Aliquots of IIMV were reconstituted and symmetrically labeled as described in chapter 3.3. Afterwards, equal volumes of labeled (proteo)liposomes and dithionite solution (10 mM final concentration) were mixed by stopped-flow, and the decrease of fluorescence intensities was monitored (chapter 3.6).

In Figure 17 kinetics of fluorescence reduction of long-chain fluorescent analogues due to chemical quenching by dithionite are displayed. Analyzing proteoliposomes labeled with either P-C12-NBD-PE or N-DP-NBD-PE, the fluorescence intensity decayed in two distinct phases (solid blue and black line in Figure 17). This was in agreement with the findings of the BSA back-extraction of NBD-labeled short-chain phospholipid analogues (chapter 4.2; 4.3). The rapid first phase reflects the fluorescence quenching of NBD-molecules that initially resided in the outer leaflet of the membrane of proteoliposomes and were therefore immediately accessible for dithionite. Phospholipid analogues residing

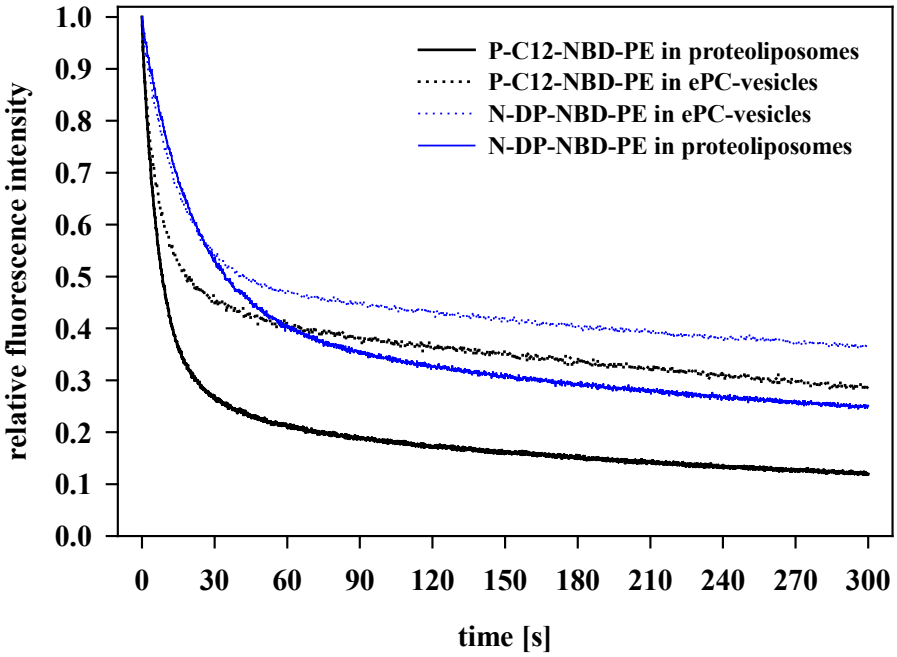


Figure 17: Influence of the chain length of analogues on the rapid transbilayer movement of fluorescent phospholipid analogues across the membrane of proteoliposomes derived from IIMV. The proteoliposomes were labeled with 0.5 mol% of the respective analogue during the reconstitution procedure (see 3.3). Equal volumes of the vesicle suspension and 20 mM dithionite were mixed in the stopped-flow accessory, and the fluorescence decrease was monitored at room temperature. The fluorescence traces represent the average of three separate kinetics. Each kinetics was normalized to the maximum fluorescence intensity.

at the lumenal leaflet of the membrane became accessible for dithionite quenching, only after they were translocated to the exoplasmic leaflet by the putative flippase, resulting in a second slower phase of fluorescence decrease (Figure 17). In the time course of the experiment (300 s), the fluorescence decreased to ~20% of the initial fluorescence intensity for the P-C12-NBD-PE and to ~30% for the head-group labeled phospholipid analogues used in this approach (Figure 17). As evident from Figure 17, the initial phase of fluorescence reduction of N-DP-NBD-PE was slower than for P-C12-NBD-PE. This indicates that the reduction of head-group labeled phospholipid analogues was possibly restricted due to steric limitations of accessibility of the NBD-group by dithionite (see discussion). Consequently, the quantitative analysis of the transbilayer movement of the fluorescent head-group labeled phospholipid analogues led only to a rough estimate (see below). Nevertheless, N-DP-NBD-PE underwent a transbilayer movement across the proteoliposome membrane. The comparison of the fluorescence traces of protein-free phospholipid vesicles (dotted blue line in Figure 17) and the corresponding proteoliposomes revealed that the presence of

bacterial proteins resulted to an enhanced reduction of fluorescence. From this, it can be concluded that long-chain, head-group labeled analogues moved from the inner leaflet to the outer leaflet of proteoliposomes facilitated by proteins and thereby became accessible for dithionite.

As evident from the fluorescence trace, the transbilayer movement of P-C12-NBD-PE (solid black line Figure 17) in proteoliposomes was very similar to that found for the short-chain phospholipid analogue M-C6-NBD-PE (compare Figure 10 and Figure 13). For protein-free liposomes (dotted lines Figure 17), which were reconstituted in parallel with proteoliposomes but in the absence of bacterial proteins, the fluorescence was quenched to more than 50% of the initial fluorescence intensity. This indicates a symmetrical distribution of the fluorescent analogues between the vesicle leaflets and the inability to cross the membrane due to the strong polar head-group. Nevertheless, a permeation of dithionite under the experimental conditions used in this assay was apparent (see below).

The quantitative analysis of transbilayer movement of long-chain and head-group labeled phospholipid analogues across the membrane of the (proteo)liposomes yielded differing rate constants as those calculated from BSA back-exchange assays (data not shown). As evident from Figure 17, the traces of the control liposomes (without bacterial proteins) did not reach a final plateau of fluorescence in the time course of the experiment. These traces followed a bi-exponential function but not a monoexponential course of fluorescence decrease. This indicates a small penetration of dithionite into the vesicles at room temperature. Although the leakage was small, the permeation of dithionite was not negligible for the estimation of rate constants by fitting. At lower temperatures (15°C), the penetration effect was eliminated (data not shown), but it can not be excluded that the decreased temperature affects the transbilayer movement of the analogues. However, as evident from Figure 17 the long-chain phospholipid analogues undergo a transbilayer movement in the presence of bacterial proteins (from 70% up to 80% fluorescence reduction on proteoliposomes compared to only ~50% - 60% on pure liposome samples).

4.6 Protein modifying treatment of reconstituted proteoliposomes

The reconstitution experiments (4.3; 4.4) revealed strong evidences that proteins involved in the flip-flop of fluorescent phospholipid analogues. To prove these findings, the influence of protein modifying substances was investigated.

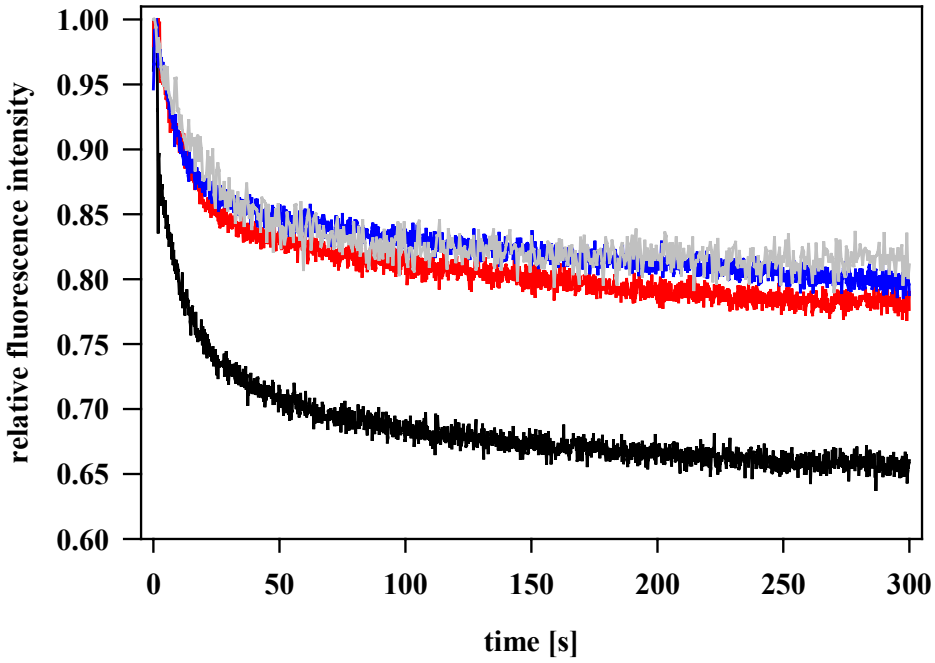


Figure 18: Kinetics of the extraction of M-C6-NBD-PE from reconstituted IIMV by BSA prior to and after proteinase K treatment. Reconstituted IIMV-derived proteoliposomes were labeled with two molpercent M-C6-NBD-PE during the reconstitution (see chapter 3.3), and a stock was treated with proteinase K (1 mg/ml) for 0 min (black trace), 30 min (red trace) and 60 min (blue trace). Each aliquot was incubated with three mM (final) PMSF for five minutes to terminate the reaction prior to analysis by stopped-flow BSA back-exchange. Traces were corrected for scattering. For comparison, the extraction kinetics measured with protein-free liposomes (gray line) is shown.

A stock of reconstituted IIMV-derived proteoliposomes labeled with two molpercent of M-C6-NBD-PE during the reconstitution was treated with proteinase K (1 mg/ml) for 0 min, 30 min and 60 min prior to analysis by stopped-flow back-exchange. The traces were corrected for scattering (see Material and Methods).

Figure 18 shows the kinetics of the extraction of M-C6-NBD-PE from reconstituted IIMV by BSA prior to and after proteinase K treatment. For comparison, the extraction kinetics measured with protein-free liposomes is shown (gray line). The amount of extractable analogues decreased dramatically after 30 min or 60 min proteinase K treatment as revealed by comparison with kinetics of untreated proteoliposomes (black line) and protein-free liposomes.

This indicates that the proteolysis eliminated protein-mediated flip-flop activity in a large fraction of the proteoliposome population after 30 min of proteinase K treatment (red line). The pool of transport-active vesicles was eliminated by the treatment with proteinase K after 60 min (blue line).

4.7 Ion exchange chromatography (IEC) with Triton extracts derived from IIMV of *E.coli*

In the preceding experiments (see chapter 4.4, 4.3, 4.6), it has been shown that proteins were involved in the transbilayer movement of fluorescent phospholipid analogues across the IIMV membrane (see discussion). In an attempt to identify the protein(s) harboring flippase activity, ion-exchange columns were used to yield protein fractions with flippase activity. 1 ml Hi Trap Q HP columns (Amersham-Pharmacia Biotech), strong anion exchangers, were utilized to separate the detergent solubilized proteins derived from *E.coli* into two fractions (flow-through and eluate) as described in chapter 3.7. After dialysis of the flow-through and the eluate proteins at room temperature, an aliquot of each fraction was reconstituted, and the resulting proteoliposomes were assayed for transport activity with the dithionite approach at 22°C. In parallel, proteoliposomes were generated from an aliquot of the total extract, i.e. from the TE, which was the starting material for the chromatographic separation of the bacterial proteins. Furthermore, ePC-vesicles were created in the absence of proteins (see chapter 3.3 for the reconstitution procedure).

4.7.1 Efficiency of the separation of proteins from *E.coli* with IEC

To test whether the IEC is a suitable tool for the separation of proteins from *E.coli* inner membranes a chromatography experiment using an anion exchange column (3.7) for fractionation was performed (see also 4.7). The resulting flow-through and eluate were analyzed by SDS-PAGE (15%) as described in chapter 3.8.

As evident from Figure 19, the experimental conditions, used for the fractionation of bacterial proteins derived from IIMV of *E.coli* with Q-Sepharose, were suitable to separate bacterial proteins by their charges. The comparison of QF (flow-through) and QE (eluate) showed striking qualitative differences in the abundance of proteins in the fractions. From the polyacrylamide gel, it was evident that more proteins were present in the eluate compared to the flow-through. Data from quantitative protein determination proved these observations.

Commonly, about two thirds of the initial protein used for separation were bound to the Q-Sepharose column (data not shown). Moreover, a number of prominent protein bands

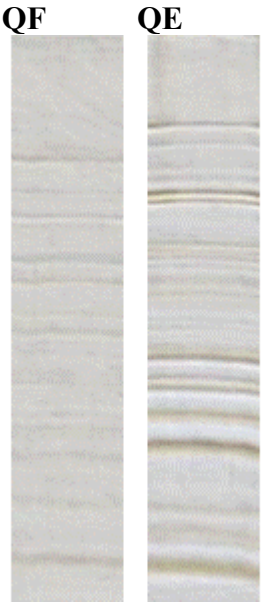


Figure 19: Qualitative analysis of proteins derived from TE of IIMV. A representative polyacrylamide gel is shown. 2.9 mg protein from solubilized IIMV (TE) were applied to an anion exchange column. The resulting flow-through (QF) and eluate (QE) were analyzed by a 15% SDS-PAGE.

in QF were not present in QE and *vice versa*. Furthermore, several bands in QF were less intensive (less protein) than in QE, which implies that proteins with similar molecular masses but different charges were separated. In summary, it can be concluded from the (qualitative) gel data and quantitative protein determinations that the proteins from the inner membrane of *E.coli* were successfully separated by their charge(s) using a strong anion exchange column.

4.7.2 Enrichment of flippase activity of inner membrane proteins of *E.coli* by anion exchange chromatography (AEC)

To analyze the phospholipid transport activity, the resulting fractions (flow-through and eluate) obtained after AEC (3.7, 4.7.1) were reconstituted in the presence of 0.5mol% M-C6-NBD-PE of the total phospholipid content as described in chapter 3.3. In parallel, proteoliposomes from the total TE (which were originally applied to the column) were prepared (3.7). Additionally, protein-

free ePC-vesicles were generated in the presence of Triton X-100 only. In order to get comparable results, the applied total amount of protein for reconstitution was similar in the different samples.

Proteoliposomes and liposomes were analyzed by the dithionite assay in cuvette experiments (3.6). Upon addition of dithionite to a suspension of liposomes, the fluorescence rapidly decreased to approximately 50% of the initial fluorescence and remained at this level (Figure 20 gray line) until 1% (w/v) Triton X-100 was added. After detergent addition, the fluorescence immediately declined to zero (data not shown). Thus, a pool of fluorescent analogues protected against dithionite reduction existed that only became accessible after membrane disruption by detergent. These data confirmed that dithionite was not permeable or the dithionite penetration was negligible, and the amount of dithionite used was sufficient to reduce all M-C6-NBD-PE present in the sample.

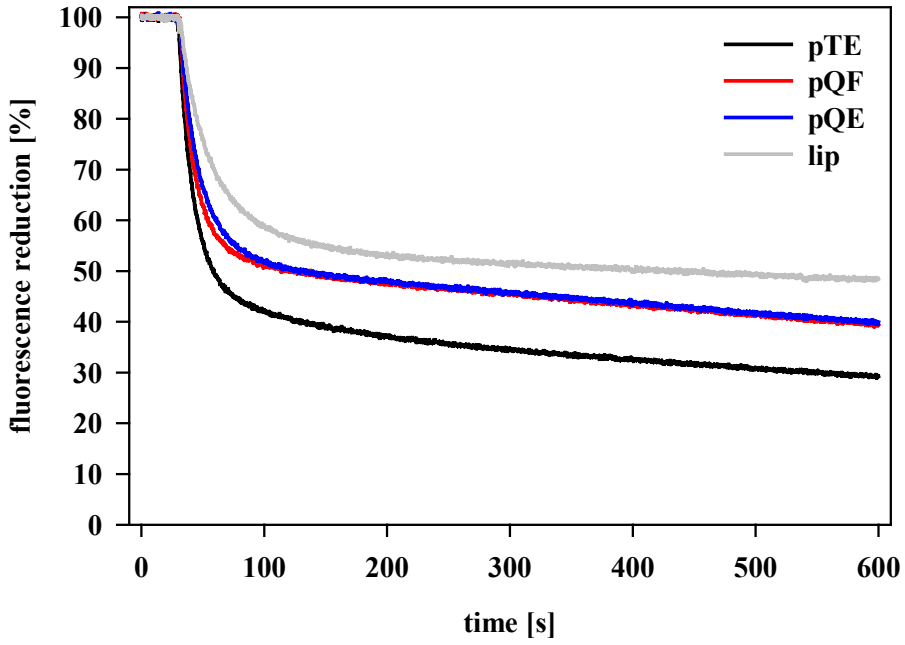


Figure 20: Comparison of flippase activity of reconstituted protein fractions after IEC. An aliquot TE (1.74mg protein) was applied to an anion exchange (Q-Sepharose) resin and the resulting flow-through (QF) and eluate (QE) were dialyzed. Fractions were taken for reconstitution to generate proteoliposomes with equal protein contents. Subsequently, the accessibility of M-C6-NBD-PE of proteoliposomes and liposomes was analyzed by the dithionite assay. 100 µl of each sample were diluted into 1.9 ml HPS in a cuvette, the fluorescence was recorded for 30 s. Then 10 mM (final) dithionite in 40 mM TRIS pH 8 was added and the resulting decay of fluorescence intensity was recorded for 600 s for each vesicle preparation. The protein concentration of the reconstituted proteoliposomes were 23.2µg/ml, 23.9µg/ml and 14.9µg/ml for pTE, pQF and pQE, respectively.

When assaying proteoliposomes, the fluorescence intensities decreased to less than 50% of the initial value (Figure 20). These data indicated that fluorescent

labeled PE, which originally resided in the inner leaflet of the proteoliposome membrane, crossed the membrane during the assay and subsequently, became accessible for dithionite. The fluorescence reduction of proteoliposomes prepared from the total protein extract (TE) and treated with dithionite was ~71%. The extent of fluorescence reduction of proteoliposomes generated from flow-through (pQF) and the eluate (pQE) of the anion exchange resin was approximately 61% (Figure 20), indicated a possibly flippase activity.

The proteoliposomes were prepared with approximately the same amounts of proteins (23.2 µg/ml, 23.9 µg/ml and 14.9 µg/ml for pTE, pQF and pQE respectively). The fluorescence traces shown in Figure 20 implicate different putative flippase activities in the proteoliposomes derived from both chromatographic fractions compared to proteoliposomes prepared from TE. The different activities were possibly due to altered protein patterns reconstituted into vesicles. As evident from Figure 20, the fluorescence traces monitored for pQF and pQE showed no differences neither in shape nor in the final fluorescence plateau, despite the two analyzed proteoliposomes classes derived from IEC fractions contained different subsets of proteins (Figure 19). This indicates that the ability of both vesicle populations to facilitate transmembrane movement of fluorescent phospholipid analogues was similar and therefore, no substantial separation of the putative flippase protein(s) under these particular conditions occurred.

Table 4: Partial purification of phospholipid flippase activity from *E.coli* inner membrane TE. The data are representative for five independent experiments.

fraction	activity A [%]	specific activity A^S [%*µmol*µg^{-1}]	protein/phospholipid ratio [µg/µmol]
TE	19.48	2.44	8.0
QF	9.36	1.11	8.4
QE	8.95	1.99	5.1

Moreover, the flippase activities found for pQF and pQE is less than that found for pTE, indicating no enrichment of flippase activity in the reconstituted fractions of anion exchange chromatography. Based on the data revealed from fluorescence measurements, the specific activities of the QF-, QE- and TE-derived

proteoliposomes were estimated, i.e. calculations of transport activities relative to the protein/phospholipid ratios as described in chapter 3.7. The results are shown in Table 4.

Indeed, the specific activity of phospholipid transport of the TE derived proteoliposomes was higher than those found for the column fractions derived proteoliposomes. Nevertheless, an approximately two-fold enrichment of flippase activity was detected in QE compared to the QF derived vesicles.

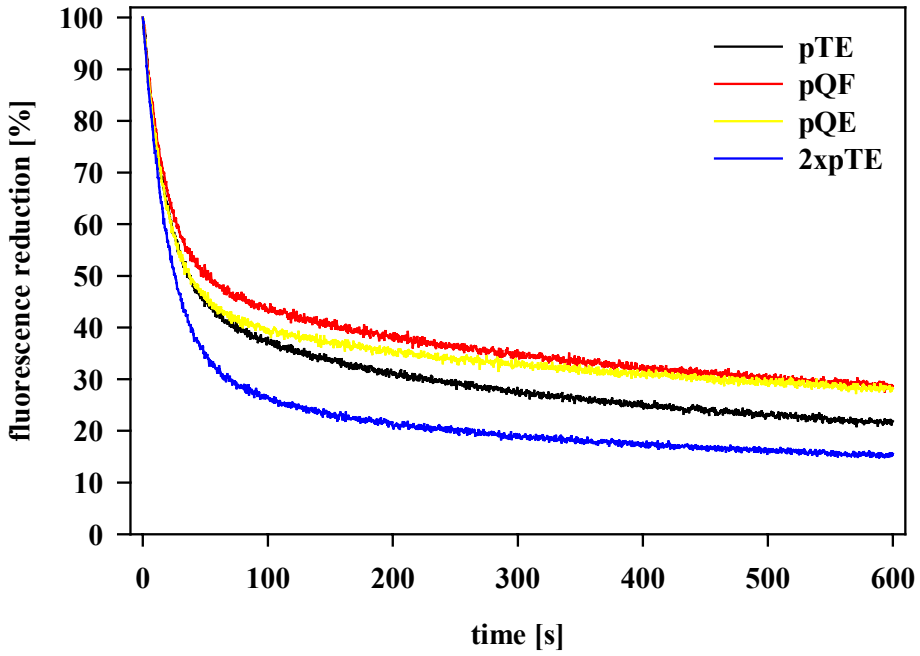


Figure 21: Comparison of flippase activities of flow-through and eluate from Q-Sepharose column and TE. An aliquot of TE was applied to an anion exchange (Q-Sepharose) resin, and the resulting flow-through (QF) and eluate (QE) were dialyzed. Proteoliposomes with equal protein contents and 0.5 mol% M-C6-NBD-PE were reconstituted. Additionally, respective amounts of the originally TE applied to the column, were taken to obtain proteoliposomes (pTE) with the same protein concentration compared to the pQF and pQE and proteoliposomes with two times higher concentration of protein (2xpTE) The protein concentrations of the proteoliposomes were 48.2 µg/ml, 50.9 µg/ml, 47.1 µg/ml and 93.2 µg/ml for pTE, pQF, pQE and 2xpTE, respectively. Subsequently, aliquots of proteoliposomes and control liposomes were analyzed by the dithionite assay (see legend of Figure **20**).

When increasing amounts of TE were reconstituted into proteoliposomes, the fluorescence reduction was greater than that seen for protein-free liposomes. The final fluorescence plateau depended on the protein/phospholipid ratio as revealed from dithionite and BSA back-extraction experiments (see chapter 4.4.1 and 4.4.2). To test, whether the protein content reconstituted from IEC column fractions and TE is sufficient for facilitated phospholipid flip-flop, a number of experiments with varying protein concentrations in the proteoliposomes from QF,

QE and TE were performed. In Figure 21 a representative experiment is displayed. The separated column fraction proteins and TE, respectively, were reconstituted into proteoliposomes containing 0.5 mol% M-C6-NBD-PE with final protein concentrations of 48.2 µg/ml, 50.9 µg/ml and 47.1 µg/ml for pTE, pQF and pQE respectively. Additionally, fluorescent labeled proteoliposomes derived from TE with an approximately doubled protein amount (93.2 µg/ml, referred to 2xpTE) were generated.

Table 5: Partial purification of phospholipid flippase activity from *E.coli* inner membrane TE. The data are representative for two independent experiments and were calculated from data shown in Figure 21.

fraction	fluorescence reduction [%]	activity A [%]	specific activity A^S [%*µmol*µg^{-1}]	protein/phospholipid ratio [µg/µmol]
TE	78.4	34.60	3.42	10.13
2xTE	84.6	40.81	1.69	24.21
QF	71.4	27.69	2.38	11.65
QE	72.0	28.27	2.15	13.16

As evident from Figure 21, the fluorescence reduction by dithionite strongly depended on the protein concentration (for comparison see also Figure 20). With increasing protein content in the proteoliposomes, an increasing number of fluorescent PE-analogues were reduced to non fluorescent species. The fluorescence traces monitored for pQF and pQE showed no differences compared to each other, and the amount of reduced analogues was lower compared to pTE (see Table 5). Moreover, proteoliposomes containing a twofold higher amount of proteins (2xpTE) showed an enhanced fluorescence reduction by dithionite (78.4% and 84.6% for pTE and 2xpTE respectively). The specific activity of 2xpTE decreased compared to pTE.

The specific activities of pQF and pQE calculated from the fluorescence measurements shown in Figure 21 were lower compared to pTE but higher than estimated for 2xpTE. Based on enhanced but similar protein contents in the proteoliposomes, no enrichment of phospholipid transport activity was detected (see 5.3). Interestingly, an enriched specific activity of phospholipid flippase

activity could not be exclusively attributed to pQF or pQE as evident from ten independent chromatographic experiments (data not shown).

4.7.3 Successive fractionation of solubilized proteins from IIMV with anion exchange chromatography

To examine the effect of ionic strength on protein separation with anion exchange resin, different elution buffers with varying salt concentrations were tested.

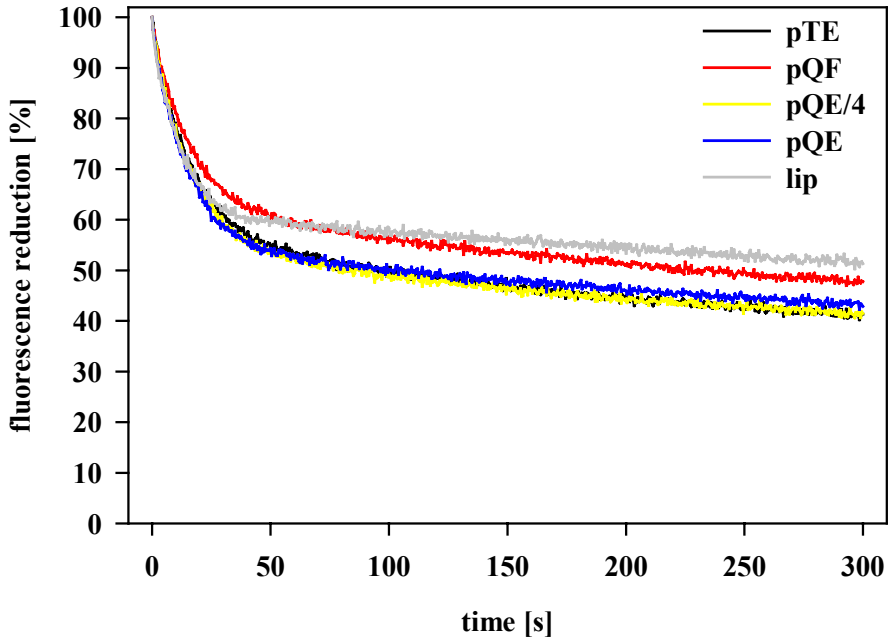


Figure 22: Flippase activities of Q-Sepharose column fractions, liposomes and TE after successive elution. An aliquot of TE was applied to an anion exchange (Q-Sepharose) resin and the resulting flow-through (QF) and eluates (QE4, QE) were dialyzed. Proteoliposomes with equal protein contents and 0.5 mol% M-C6-NBD-PE were generated. For comparison a respective amount of the originally TE applied to the column was reconstituted (pTE) to create proteoliposomes with the same protein concentration compared to the fractions. The protein concentration of the proteoliposomes were 22.8 µg/ml, 15.3 µg/ml, 14.4 µg/ml and 23.5 µg/ml for pTE, pQF, pQE4 and pQE, respectively. Subsequently, aliquots of proteoliposomes and control liposomes were analyzed by the dithionite assay. To this end, 100 µl of sample were diluted in 1.9 ml 10 mM HPS and the fluorescence decrease by reduction with 10 mM dithionite was monitored for 300 s using the stopped-flow accessory. All experiments were carried out at room temperature.

For this reason, aliquots of TE were loaded onto a 1 ml Hi Trap Q HP column (Amersham-Pharmacia Biotech) and passed over the column equilibrated with buffer B. Subsequently, the column was washed with buffer A and the wash and flow-through were pooled. Bound proteins were first eluted with two

milliliters modified buffer D* (buffer D with 0.25 M NaCl instead of 1 M NaCl) and finally eluted with two milliliter buffer D to yield two distinct elution fractions. The column fractions were dialyzed against 10 mM HPS (pH 7.5) containing 0.2% Triton X-100 for 1.5 h at room temperature.

Aliquots of the flow-through and the eluates were reconstituted into ePC vesicles as described in 3.3 and 3.7 to generate M-C6-NBD-PE (0.5 mol%) containing proteoliposomes with approximately similar concentrations of protein. The fluorescence measurements were performed as outlined in chapter 3.5.2.

In Figure 22 a representative experiment is displayed. The final fluorescence intensity of the control liposomes (pure lipid vesicles) leveled off at approximately 51% of the initial fluorescence (Figure 21, gray line). The fluorescence reduction of the proteoliposome samples amounted to more than 50%, indicating that short-chain phospholipid analogues of PE redistributed from the lumenal side of the membrane and became accessible to dithionite quenching. As evident from Figure 22, the flippase activity was different depending on the samples probed. The final fluorescence reduction measured for pQF (59.5% of initial intensity) was less than those monitored for pQE4, pQE and pTE (64.1%, 62.3% and 65.4% respectively), indicating slightly different flippase activities in the vesicles due to differences in the protein composition of the proteoliposomes.

Table 6: Flippase activity in fractions of anion exchange chromatography. The data are representative for three independent experiments. pTE refers to proteoliposomes prepared from TE of IIMV. pQF, pQE4 and pQE designated for proteoliposomes reconstituted from AEC separated proteins of the flow-through and eluates, respectively.

	fluorescence reduction [%]	**activity A [%]**	**specific activity A^S [%*µmol*µg^{-1}]**	**protein/phospholipid ratio [µg/µmol]**
pTE	65.4	16.66	2.03	8.19
pQF	59.5	10.75	2.20	4.89
pQE4	64.1	15.42	1.92	8.04
pQE	62.3	13.55	1.89	7.16

Based on these measurements, the specific activities were calculated to normalize the phospholipid flippase activity to the protein content in the proteoliposomes (3.7). The specific activity of QF derived proteoliposomes is greater than those for all other samples analyzed, although the protein content in

this fraction is lower compared to TE, QE4 and QE (Table 6). Nevertheless, the flippase activity was not significantly enriched in pQF after elution with varying ionic strength.

5 Discussion

In the first part of this thesis, we characterized the transmembrane movement and transverse distribution of fluorescent phospholipid analogues across the inner membrane of *E.coli*. For this analysis, we used isolated IIMV and reconstituted proteoliposomes from detergent extracts of inner membrane vesicles (IIMV) of *E.coli*. To determine the transbilayer movement of phospholipids, a recently developed stopped-flow BSA back-exchange assay was established. We could show that this new approach is also applicable for IIMV and reconstituted systems.

We found that the transbilayer movement of the analyzed phospholipid analogues across IIMV membranes was head-group independent and very rapid. We observed that the rapid flip-flop of phospholipid analogues was restored in reconstituted vesicles from detergent extracts of IIMV. Moreover, a rapid transbilayer movement of fluorescent long-chain PE analogues was found. Our investigations revealed a strong requirement of proteins for the rapid transmembrane movement of phospholipid analogues.

In the second part of the thesis, we aimed at purifying the proteins responsible for the observed protein dependent flip-flop of fluorescent phospholipid analogues. To isolate the putative flippase(s) we used ion exchange chromatography. To our surprise, we were not able to enrich specific flippase activity in any of the analyzed fractions by this method, indicating that flippase activity is not mediated by one specific protein but rather by at least two distinct facilitators or by the presence of proteins within the membrane.

5.1 Transbilayer movement of short-chain, fluorescent phospholipid analogues in IIMV and reconstituted proteoliposomes derived from IIMV

To investigate the transmembrane movement of short-chain fluorescent phospholipid analogues across the inner membrane of *E.coli*, we combined the BSA back-exchange assay with a stopped-flow technique. This combination allowed us to directly record fluorescence changes that occurred during the extraction of fluorescent labeled phospholipid analogues from IIMV and proteoliposomes with high time resolution. Fluorescence kinetics were analyzed via the three-compartment model (Marx, et al., 2000), to deduce the rate constants (and respective half-times) of transbilayer movement, and the distribution of phospholipid analogues in the two leaflets of the bilayer.

To ensure that approaches used in this study for the determination of transmembrane movement of fluorescent phospholipid analogues were appropriate, a series of experiments were carried out to demonstrate that i) the analogues are equilibrated between both leaflets of IIMV at the beginning of the BSA back-exchange stopped-flow assay, and ii) BSA extracts all analogues in the time course of the assay. Otherwise, the estimation of half-times of transmembrane movement of analogues, and their transbilayer distribution would not have been possible. As shown in chapter 4.2 (Figure 8 and Figure 9), these preconditions were fulfilled by the chosen experimental set-up.

The applicability of the stopped-flow BSA back-exchange approach depends on the quantitative relation between the rate constants for the extraction of analogues by BSA, and the rate constants for the transbilayer movements of analogues. The latter can be measured by this approach only, if the extraction step is significantly faster compared to the transmembrane movement of the analogues. Apparent from the initial rapid fluorescence decline and the subsequent slower phase of fluorescence decrease, this assumption holds true in our system. For example, we found half-times of fluorescent analogues extraction of fluorescent PE analogues from protein-free liposomes by BSA of about eight seconds (see chapter 4.3). On the other hand, the half-times of transbilayer movement of fluorescent PE in IIMV membranes were about 1-3 min. Therefore, the rapid

initial fluorescence decline represents the extraction of analogues by BSA from the outer leaflet and the second slower phase of fluorescence decrease reflects the transbilayer movement of the fluorescent analogues. The observed half-time of about five seconds for the initial outer leaflet extraction of analogues from IIMV was similar to those previously described for microsomes from rat liver cells (Marx, et al., 2000).

The half-times of movement of fluorescent PE and PC analogues to the periplasmic leaflet were about 3-4 times slower than those observed for the movement to the cytoplasmic layer. About 23% of the PE analogues and 18% of the PC analogues were located in the periplasmic leaflet of the vesicle membranes. However, it is unlikely that this asymmetric distribution of the analogues corresponds to the *in vivo* distribution across the inner membrane of *E.coli* cells. This is particularly true for the PE analogues. PE is not only the major phospholipid of the inner membrane, it also has to be conveyed to the outer membrane to populate the periplasmic leaflet of the outer membrane. Furthermore, PE is utilized for modifications on periplasmic oligosaccharides and protein modification (Huijbregts, et al., 1996; Sankaran and Wu, 1994). Huijbregts *et al.* speculated that the consumption of PE by intracellular processes for the above mentioned modifications or by integration of PE into the outer membrane provides a naturally sink for phospholipid transport processes *in vivo* (Huijbregts, et al., 1996). However, isolated IIMV from *E.coli* lack these processes and therefore, lack the need for substantial amounts of PE in the periplasmic leaflet, which could partially explain our findings. Possibly, the lack of such a sink resulted in the loss of an additional trigger for phospholipid synthesis and subsequently, in a difference of the protein mediated transmembrane equilibration of phospholipids *in vivo* and *in vitro*. Nevertheless, Huijbregts *et al.* showed that newly synthesized radioactive labeled PE preferentially redistributes to the periplasmic leaflet (65%) (Huijbregts, et al., 1998). The discrepancy to our findings might be related to the chemical structure of the used analogues, causing them to behave differently from their endogenous counterparts in *in vitro* systems. Therefore, a specific transbilayer distribution of phospholipids may occur, which was different in whole cells compared to isolated IIMV. Moreover, it is not known how the isolation procedure of IIMV affects the

transbilayer distribution of endogenous phospholipids. Additionally, the inverted structure of the isolated inner membrane vesicles and the relative abrasive isolation method possibly led to the loss or shielding of protein components in IIMV, which are important for the equilibration of phospholipids across the inner membrane in intact cells.

The transbilayer movement of short-chain, fluorescent PC (M-C6-NBD-PC) analogues was similar to that of the PE analogues. Interestingly, although PC is not present in the envelope of *E.coli*., we found that a fluorescent analogue of PC moved rapidly across the IIMV bilayer. This is consistent with previous findings that this process is not head-group specific (Bishop and Bell, 1985; Herrmann, et al., 1990; Huijbregts, et al., 1996).

The transbilayer distribution of the short-chain fluorescent PG analogues (M-C6-NBD-PG) was ~40% at the periplasmic leaflet and ~60% at the cytoplasmic leaflet of the vesicle membranes and therefore, close to a symmetrical distribution across the membrane of IIMV. The almost symmetrical transmembrane distribution indicates that PG has a different transverse membrane distribution in *E.coli* compared to PE. The transmembrane movement of the used PG analogues is also different from that of PS and PC analogues. While the half-times for outward movement of the PG analogues (~65 s) were similar to those found for fluorescent PE and PC analogues (~53 s and 40 s, respectively) the inward movement was faster as found for PE, PC and PS analogues. Additionally, inward movement of PG was 1.5 times slower than outward movement of PG compared to PC and PE analogues. This also indicates that PG phospholipid analogues redistribute different across the IIMV membranes, possibly facilitated by a different protein.

The rapid transmembrane movement of fluorescent analogues, which we found in this study, is in agreement with previously reported results (Huijbregts, et al., 1996). Using the dithionite assay, Huijbregts *et al.* (Huijbregts, et al., 1996) observed that the velocity of transmembrane movement of short-chain, fluorescent labeled phospholipid analogues across IIMV membranes at 37°C was in the same order of magnitude (about seven minutes). The same group reported half-times of redistribution of endogenously synthesized radioactive labeled PE analogues of about one minute in both IIMV and right-side out vesicles from *E.coli*

(Huijbregts, et al., 1998). However, the authors reported a conversion of the PG analogue to a fluorescent cardiolipin (CL) (Huijbregts, et al., 1996). The transmembrane movement of this CL analogue was found to be 2.4 times slower than that of the fluorescent short-chain PG analogue. Huijbregts *et al.* concluded that structural differences like head-group charges and fatty acid composition possibly led to different transbilayer movement (Huijbregts, et al., 1996). The data presented here do not support this hypothesis. As evident from TLC analysis of IIMV labeled with fluorescent short-chain PG, CL was not present in the IIMV membrane (data not shown), indicating that no conversion of PG to CL took place in the time course of experiment.

To investigate the transbilayer movement of fluorescent phospholipid analogues in more detail, we established a reconstitution assay. After detergent solubilization of IIMV, fluorescent phospholipid analogues (M-C6-NBD-PE or M-C6-NBD-PG) were added, and the detergent was removed resulting in equably labeled proteoliposomes. In parallel, we created liposomes without bacterial proteins but in the presence of detergent during reconstitution. While the rapid initial fluorescence decay was similar between protein containing and protein-free vesicles, only protein containing proteoliposomes showed a second slower phase of fluorescence decay similar to that of IIMV. So far, we were able to reconstitute the flip-flop without significant impairment.

Our investigations of the transbilayer movement and transverse distribution of fluorescent PE analogues across reconstituted proteoliposomes, confirmed the results that we obtained from BSA back-exchange kinetics with IIMV. Nevertheless, the reconstitution experiments revealed that the fluorescent PG analogues behaved different compared to the respective PE analogues. Almost nothing is known about the transbilayer distribution in bacterial membranes. As already outlined above, PG possibly has a different transbilayer distribution compared to PE in *E.coli* cells, and phospholipids in general are substrates for many metabolic enzymes. For example, in one study it has been demonstrated that pss was regulated by the presence of PG (Saha, et al., 1996). Using artificial transmembrane peptides, Kol *et al.* reported a coherence of the presence of PG and PE in order to regulate the transmembrane transport of phospholipids across

vesicle membranes (Kol, et al., 2003). Additionally, anionic lipids in the *E.coli* inner membrane were found to localize positively charged membrane protein segments to the cytoplasmic side of the membrane (van Klompenburg, et al., 1997). In turn, this mechanism possibly regulates the orientation of fluorescence PG analogues. Therefore, it is possible that PG has distinct lateral and transverse distribution at the inner membrane of *E.coli*, which is different from that of PE. Thus, transbilayer distribution of PG might be regulated by different (additional) protein dependent mechanisms. However, both phospholipid analogues underwent a rapid transbilayer movement across reconstituted proteoliposomes, which was protein dependent.

In reconstituted proteoliposomes as well as IIMV, we found a faster outward movement of analogues compared to the inward movement. Assuming that ATP-independent flippases work bi-directional, the rate constants in reconstituted vesicles should be similar for inward and outward movement. In particular, since the specific transbilayer orientation of membrane proteins in IIMV was not preserved in reconstituted proteoliposomes. The reason for the observed differences is unclear. A possible explanation for our findings is that the protein dependent flip-flop of phospholipids is facilitated by more than one protein, one of which was not accurately incorporated into the membrane or partially damaged. Supportive of this explanation are findings by Menon *et al*.. They observed that at least two different proteins were able to facilitate phospholipid transmembrane movement (Menon, et al., 2000). It also has to be taken into account that the experimental set-up, with large excess of BSA on the outside of the vesicles, somehow biased the results to give a faster outward movement. We can also not preclude that the amount of analogues in the outer leaflet of proteoliposomes was overestimated, since a small residual pool of fluorescent analogues (<10%) could not be extracted by BSA. Presumably, these non-extractable analogues stacked on the inner leaflet of vesicles lacking a rapid flip-flop activity.

To prove our findings of the BSA back-exchange assay, we performed a dithionite assay (4.4.2). Using this assay, the half-time of the outward movement of fluorescent labeled PE analogues in reconstituted proteoliposomes derived

from IIMV (~40 s) were very similar compared to those calculated from the BSA back exchange assay (~30 s). The calculated half-times of outward movement were slower than those found by BSA back-extraction due to slow dithionite penetration. The permeation of dithionite could not completely prevented. Thus, the half-times were slightly overestimated by our three-compartment model, since the slow penetration of dithionite affected the fluorescence reduction kinetics. However, the kinetic data from the reduction assay were coherent with the findings of the BSA back-extraction assay.

5.2 Effect of proteins on the transport of fluorescent phospholipid analogues

E.coli is a rapidly growing organism, dividing once every half hour. This requires rapid synthesis of new membrane, and consequently, a rapid transbilayer movement of newly synthesized phospholipids. For a bilayer membrane, expansion of one monolayer with respect to the other causes curvatures and extrusion, and eventually vesiculation, a process which can be easily rationalized in the frame of the bilayer couple model (Sheetz and Singer, 1974). Therefore, to preserve the stability of the inner membrane of *E.coli*, rapid redistribution of phospholipids is of importance. In vesicles composed only of lipids from the inner membrane of *E.coli* transbilayer movement of phospholipids was slow (data not shown) and as previously shown for pure lipid membranes (Kornberg and McConnell, 1971). Thus, proteins acting as a flippase have been assumed to mediate efficient phospholipid flip-flop. According to available data, flippase activities are typical for phospholipid synthesizing membranes of bacteria (Hrafnsdottir and Menon, 2000; Huijbregts, et al., 1996; Huijbregts, et al., 1998) and eukaryotic cells (Buton, et al., 1996; Herrmann, et al., 1990; Marx, et al., 2000; Menon, et al., 2000; Nicolson and Mayinger, 2000). Indeed, in the ER of eukaryotic cells such as rat liver cells (Marx, et al., 2000) as well as yeast cells (Marx, U. and Herrmann, A., unpublished observation) a rapid protein-dependent transbilayer movement has been unequivocally demonstrated. As it was shown previously by the stopped-flow approach, the half-times of the flip-flop of short-chain, fluorescent PC and PE in microsomes (Marx, et al., 2000) were in the same order as found here for IIMV and proteoliposomes.

To elucidate the role of proteins in the transbilayer movement of phospholipid analogues, the reconstitution assay was used. A number of vesicles with different protein to phospholipid ratios were created and the resulting proteoliposomes were studied by the stopped-flow BSA back-extraction assay. With increasing amounts of protein (up to 100µg/ml), the fluorescence decline became stronger (Figure 13). In proteoliposomes containing more than ~100µg/ml protein, we observed no substantial increase of flippase activity compared to proteoliposomes containing less than 100µg/ml protein. As evident from Figure

13 B and Figure 14, we found that vesicles with a high protein content (>100µg/ml) showed an activity maximum of the putative flippase. Thus, in a vesicle population prepared with ~100 µg/ml protein, all vesicles were equipped with a putative flippase. As deduced from the rate constants (see legend to Figure 13), the kinetics of transbilayer movement of fluorescent PE analogues in proteoliposomes containing proteins in the range 0-100 µg/ml (Figure 13 B, Figure 14) was essentially independent of protein content. This suggests that in this range of protein content the proteoliposome population included some vesicles with at least one flippase and some vesicles lacking transport mediating protein(s).

Experiments carried out with proteoliposomes containing different amounts of protein and probed with dithionite (Figure 16) confirmed our findings from the BSA back-exchange assay (Figure 13 and Figure 14): (i) the transmembrane movement of fluorescent PE analogues was strongly protein-dependent, and (ii) at a distinct protein quantity every vesicle must have contained at least one functional facilitator of transbilayer flip-flop of PE analogues. We observed that beyond the inflection point (~80 µg/ml) of the dose response plot shown in Figure 16, no enhancement in transport activity occurred. This inflection point was very similar to the inflection point (~100 µg/ml) we had determined by the BSA back-exchange assay.

The dose response plot in Figure 14 can be used to calculate the abundance of flippase among solubilized and reconstituted IIMV proteins. At the inflection point of the dose response plot with a protein to phospholipid ratio of ~35 µg/µmol (corresponds to 100µg/ml, see Figure 14), on average each proteoliposome is expect to contain a single flippase. For samples prepared at >35 µg/µmol, vesicles contain one or more flippases per vesicle. Using this figure and assuming that the mean molecular mass of IIMV membrane proteins is ~50kDa, each vesicle prepared at 35 µg/µmol contains 300 protein molecules on average. This implies that the abundance of functional flippases is about 1 to 300 or 0.33% by weight of the reconstituted IIMV membrane proteins. This estimate is similar to the previously described 0.2% (Menon, et al., 2000) and 0.15% (Gummadi and Menon, 2002), which were deduced from experiments with rat

liver ER membrane proteins, as well as the estimate of 0.6% obtained in an earlier study on rat liver microsome membranes (Backer and Dawidowicz, 1987).

Further supportive of the protein dependence of the fast transbilayer movement of fluorescent analogues were our findings regarding proteinase K treatment of proteoliposomes. We observed that the transport activity in proteoliposomes was eliminated after 60 min of proteinase K treatment. Proteinase K hydrolyzes peptide bonds exposed to the aqueous milieu and leaves the transmembrane segments untouched. Since the rapid flip-flop of phospholipid analogues was almost eliminated, it is likely that the putative flippase(s) must contain ectodomains, which are important for the phospholipid transmembrane redistribution. Moreover, our data suggest that transmembrane segments alone are not sufficient for the facilitated flip-flop of phospholipids across the vesicle membrane (for further discussion see 5.4).

5.3 Ion exchange chromatography - Attempts to enrich flippase activity

In this thesis, we showed that proteins were involved in rapid flip-flop of fluorescent phospholipid analogues across IIMV derived membranes (4.3; 4.4). The question whether specific protein(s) were responsible for the rapid transbilayer movement of phospholipids or the rapid flip-flop is a consequence of the presence of membrane proteins remained unclear.

To investigate this question in more detail, we separated transmembrane proteins by their distinct properties, in particular, by their charges. We utilized anion exchange chromatography (AEC) to separate distinct fractions from a total IIMV protein extract of *E.coli.* After AEC, protein mixtures were yielded, which were less complex compared to the total protein content. After separation, fractionated proteins were reconstituted into proteoliposomes and we analyzed the ability of selected protein fraction to mediate rapid flip-flop of phospholipids across the membranes. If specific proteins are required for the rapid phospholipid flip-flop, it should be possible to enrich this transport activity by protein fractionation.

The transbilayer movement of fluorescent PE analogues across reconstituted proteoliposome membranes derived from these fractions was investigated. A prerequisite for this analysis is the generation of proteoliposomes with approximately similar protein content to allow comparison of flippase activities between different fraction samples (see chapter 4.4).

We applied TE from IIMV to a strong anion exchange resin (Q-Sepharose). The resulting flow-through (QF) and eluate (QE) were reconstituted into proteoliposomes and analyzed by the dithionite assay (3.6). Both reconstituted protein fractions, pQF and pQE showed flippase activity compared to control liposomes. However, as revealed from the fluorescence traces, pQF and pQE exhibited only minimum differences in fluorescence reduction. This indicates that AEC was not efficient to enrich the putative flippase. It is also possible that more than one putative flippase exists. If the distinct flippases contain different biochemical and structural properties, they could be hardly separated by AEC under the conditions used in our approach.

However, if only one type of a putative flippase exists, the experimental conditions we used were possibly not sufficient to separate this protein in one fraction. To exclude that the protein quantity impaired our assay, we carried out experiments with higher protein concentrations in the proteoliposomes (Figure 21) but in the linear range of the dose-response plot (see Figure 13 and Figure 14). We observed no differences in specific activities compared to lower protein content reconstituted into proteoliposomes. The phospholipid flippase activity of reconstituted fractions was lower compared to control proteoliposomes prepared from the total TE of IIMV at similar protein concentration (Figure 20 and Figure 21).

To improve the efficiency of protein fractionation, several conditions were tested. For example, successive elution, i.e. bound proteins were discontinuously eluted with buffers of increasing ionic strength. This resulted in only slightly enriched phospholipid flippase (specific) activity in pQF (Figure 22 and Table 6). Apparently, the stepwise elution diluted the transport activity originally found in the eluate QE into two fractions (QE4 and QE). Consequently, since the fluorescence kinetics and calculations on the specific activities revealed no enrichment of flippase activity in a specific anion exchange column fraction, we found no indications whether specific flippases were responsible for flip-flop of fluorescent PE. Nevertheless, nothing is known about the structure of the putative bacterial flippase. It can be a simple protein spanning the membrane only once or a complex protein with several transmembrane domains and/or various subunits. Hence, it cannot be ruled out that subunits or cofactors necessary for the efficient rapid phospholipid flip-flop were inactivated and/or dispersed into different fractions by the strong anion exchange chromatography procedure, but were present in proteoliposomes generated from total TE of IIMV.

Hrafnsdottir and Menon showed that chromatographic fractionation of a detergent extract from *Bacillus subtilis* cell membranes resulted in populations of vesicles showing different specific activities (Hrafnsdottir and Menon, 2000). Furthermore, they found by glycerol gradient analysis an enriched transport activity in a distinct fraction, which sedimented at ~4S, correlating with the presence of specific proteins involved in transmembrane movement of phospholipids (Hrafnsdottir and Menon, 2000).

Partial purification and characterization of putative flippase proteins in the ER of rat liver microsomes were reported by Gummadi *et al.* and Menon *et al.* (Gummadi and Menon, 2002; Menon, et al., 2000). Menon *et al.* clearly demonstrated that the transport of a synthetic PC across the ER membrane was facilitated by specific proteins. After glycerol gradient fractionation and IEC they presented data, consistent with the idea that two transporter are capable of affecting the bi-directional translocation of phospholipids (Menon, et al., 2000). Moreover, Gummadi and Menon were able to enrich the ER flippase activity up to 15-fold, after TE samples from rat liver microsomes, which were passed sequentially over an weak anion and over a weak cation exchange resin (Gummadi and Menon, 2002). This implies that, very likely, specific proteins are involved in the rapid, energy independent transbilayer transport of phospholipids. However, our data indicates that different strategies are needed to purify flippase proteins from *E.coli* inner membranes.

5.4 Are specific proteins required for phospholipid flip-flop?

The molecular nature of the flippase remains to be determined. We showed that the phospholipid transbilayer movement was independent of the head-group of phospholipids, as tested with fluorescent analogues of PE, PG, PS and PC. This was also observed in the ER membrane of rat liver cells (Buton, et al., 1996; Herrmann, et al., 1990; Marx, et al., 2000).

It is evident that these proteins must possess domains that enable a rapid redistribution of phospholipids between the two leaflets of a membrane bilayer. The model of Kol *et al.* postulates that the accumulated weak effects of many transmembrane helices were sufficient to allow phospholipid flip-flop in biogenic membranes (Kol, et al., 2001; Kol, et al., 2003). They found that phospholipid flop mediated by transmembrane peptides in model membranes, was modulated by the lipid composition (Kol, et al., 2003). Furthermore, they observed that the transmembrane movement of phospholipids was head-group dependent and argued this was due to the distinct charge pattern of the lipid species. It is unlikely that transmembrane domains *per se* are sufficient to mediate a fast flip-flop. Otherwise, a fast transbilayer movement of phospholipids would be a typical feature of all biological membranes. This is not the case. Our findings substantiated observations that appreciable levels of phospholipid flip-flop occurred in biogenic membranes. Furthermore, protease treatment led to a striking decrease of phospholipid transmembrane movement across reconstituted IIMV derived proteoliposome membranes. Since, proteinase K treatment leaves transmembrane domains intact, this indicates that transmembrane domains are not the only determinant of transbilayer movement of phospholipids.

Assuming that rapid phospholipid flip-flop is a general, essential property of biogenic membranes and that each of those membranes has a characteristic lipid assembly depending on the organism, it is unlikely that newly synthesized (phospho)lipids were transported due to their charges. A more reasonable explanation is that specific protein(s) facilitate transbilayer movement, possibly, under control of specific co-factors like negatively charged (e.g. PG) or non-bilayer forming lipids (e.g. PE). For example, as mentioned above, such a regulatory mechanism is known for the pss, a major enzyme in the PE synthesis in

E.coli: This enzyme is up-regulated by the presence of PG (Saha, et al., 1996), leading to a specific transverse and/or lateral membrane distribution. Kol *et al.* found that flop of short-chain fluorescent labeled PG was inhibited by increasing PE concentrations and stimulated by increasing the fraction of PG (Kol, et al., 2003). The hypothesis made by Kol *et al.* that this could be an autoregulatory mechanism (Kol, et al., 2003) is supported by our observations, that PG exhibited a different transmembrane distribution in reconstituted vesicles compared to PE.

It is obvious that transmembrane distribution is a very important process and needs to be regulated. In consequence, it is very likely that a protein mediated tuning of lipid distribution is necessary for every cellular membrane in particular for biogenic membranes with regards to the specific function of the individual membranes.

6 Future perspectives

Despite the documentation of rapid phospholipid flip-flop in biogenic membrane systems like bacterial membranes or the ER of eukaryotes, and a variety of persuasive evidence for protein involvement in this process, no biogenic flippase has been isolated and the mechanism(s) of catalyzed flip-flop remain(s) to be described. Previous problems of an adequate assay for such very rapid processes have been overcome with our stopped-flow BSA back-exchange assay which provides an adequate time resolution to measure flippase activity in biogenic membranes.

In order to purify the putative flippase protein(s), we utilized anion exchange chromatography. Surprisingly, we were not able to yield a fraction of *E.coli* inner membrane proteins, which exhibited an enhanced flip-flop activity after reconstitution of these proteins into eggPC proteoliposomes. The combination of ion exchange chromatography with either glycerol gradient fractionation or other chromatographic methods (e.g. size exclusion, two-dimensional gel electrophoresis) could provide stronger evidences for an involvement of (a) specific protein(s) in the phospholipid flip-flop across biogenic membranes.

Another possibility to identify the putative flippase would be a rather theoretical molecular approach. The entire genomes of several bacteria (e.g. *E.coli*, *Haemophilus influenzae*, *Mycobacterium tuberculosis*) and that of *S. cerevisiae* are known. Using data base screening, it might be possible to reduce the number of possible candidates, which facilitate the energy independent phospholipid flip-flop. For example, access to the complete, annotated *E.coli* genome in several data bases allows the specific screening for transmembrane domains. Many of them are associated to ABC domains and were not relevant for the ATP independent transport. In a step by step procedure all transporter with e.g. known function etc. can be discarded. Thus, it should be possible to identify proteins with transmembrane domain(s) and unknown function and specifically investigate these candidates. Additionally, it would be possible to investigate the involvement of strong candidates in the lipid flip-flop in more detail using molecular biological and genetically methods, e.g. by generating point mutations.

Possibly, temperature sensitive mutants provide another tool to research the transbilayer movement of (phospho)lipids in biogenic membranes.

7 Summary

In the plasma membrane of bacteria, phospholipids are synthesized on the cytoplasmic leaflet of the plasma membrane. To ensure balanced growth and thus, stability of biogenic membranes, half of the newly synthesized lipids must move to the opposing leaflet. It is known that this phospholipid transmembrane movement (flip-flop) is rapid, head-group independent and possibly protein mediated. However, the exact mechanism of this process remains elusive.

To investigate these fundamental transbilayer phospholipid transport processes in biogenic membranes, a novel stopped-flow BSA back-exchange assay was utilized to characterize the transmembrane movement and transbilayer distribution of fluorescent labeled, short-chain phospholipid analogues in *ex vivo* membranes. This approach is based on stopped-flow fluorescence spectroscopy, and the fact that BSA is able to extract fluorescent labeled, short-chain phospholipid analogues from the outer leaflet of (bio)membranes. We chose isolated inverted inner membrane vesicles (IIMV) derived from *E.coli* wild type MG1655, both for their simple membrane organization and for their suitability as a simple model organism for phospholipid flip-flop.

We observed that fluorescent-labeled, short-chain analogues of the major phospholipids in *E.coli*, phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), rapidly redistributed across the IIMV bilayer with half-times of less than three minutes. Furthermore, fluorescent, short-chain phospholipid analogues of phosphatidylcholine (PC) and phosphatidylserine (PS), which are not naturally occurring phospholipids in *E.coli* membranes, behaved similar to the PE and PG analogues. Surprisingly, we found that at equilibrium all fluorescent analogues were asymmetrically distributed between the two leaflet of the IIMV membranes. Approximately 23% of the PE, 18% of the PC, 34% of the PG and 26% of the PS analogues were located in the cytoplasmic leaflet. In conclusion, our analysis showed that the transmembrane movement of the phospholipid analogues across the membrane of IIMV was very rapid, bi-directional and head-group independent. These results were confirmed by an alternative fluorescence quenching assay, which is based on the chemical reduction of the fluorescence by dithionite. Analysis of proteoliposomes, containing fluorescent, long-chain or

fluorescent head-group labeled PE analogues revealed that the chain length did not influence the rapid flip-flop of phospholipid analogues.

To analyze the relevance of proteins for the transmembrane movement of fluorescent analogues, we measured flip-flop of phospholipid analogues in untreated and proteinase K treated vesicles generated from protein detergent extracts of IIMV. We found that the rapid transbilayer movement of phospholipid analogues across the membrane was maintained in untreated reconstituted vesicles. However, the flip-flop of fluorescent PG and PE analogues was eliminated in proteinase K treated vesicles. To further characterize this protein dependency, we reconstituted vesicles with increasing amounts of IIMV proteins. While we detected no flip-flop in protein-free liposomes, we observed that in reconstituted proteoliposomes containing more than 100 μg/ml of IIMV proteins, the flip-flop of short-chain analogues of PE and PG was as rapid as we found in isolated IIMV (half-times less than 2 min). We also observed that all fluorescent analogues were extracted from proteoliposomes containing more than 100 μg/ml of the IIMV proteins, similarly to what we found in IIMV. However, the amount of extractable fluorescent phospholipids analogues correlated with the amount of protein reconstituted into the proteoliposomes, strongly indicating, that protein concentrations below 100 μg/ml were not sufficient to equip every vesicle with proteins that facilitate the transmembrane movement of the fluorescent analogues. These data clearly demonstrated that the transmembrane movement of phospholipids must be facilitated by (a) protein(s).

To identify the molecular basis of the protein-mediated, rapid transmembrane movement of phospholipids across IIMV membranes, we used ion exchange chromatography (IEC) to separate the IIMV proteins. Detergent extracts from IIMV were applied on a strong anion exchanger, and the resulting fractions were reconstituted separately into proteoliposomes. To our surprise, we did not observe an enhanced flip-flop activity in any of the fractions, indicating that at least two proteins with possibly opposite netto charges or several subunits, which were not separable by AEC, are involved. Further analysis using different protein separation techniques will be necessary to identify the putative flippase complex.

Nevertheless, the presented data supplies strong evidence, that the bi-directional transmembrane movement of phospholipids is protein mediated, head-group and ATP independent.

8 Zusammenfassung

In Bakterien werden Phospholipide auf der cytoplasmatischen Seite der Plasmamembran synthetisiert. Damit ein gleichmäßiges Wachstum und somit die Stabilität biogener Membranen, d.h. Membranen, an bzw. in denen Lipidsynthese stattfindet, gewährleistet ist, muss zumindestens die Hälfte neu synthetisierter Lipide auf die entgegengesetzte Membranhälfte gelangen. Aus früheren Untersuchungen ist bereits bekannt, dass dieser transversale Phospholipidaustausch, auch als Flip-Flop bezeichnet, sehr schnell, kopfgruppenunabhängig und möglicherweise proteinabhängig ist. Dennoch sind die genauen Mechanismen dieser Prozesse noch weitgehend unverstanden.

Um die oben erwähnten grundlegenden Phospholipidtransportprozesse zwischen beiden Membranhälften genauer untersuchen zu können, wandten wir einen neuartigen, sogenannten stopped-flow BSA back-extraction Assay an. Mit Hilfe dieses Assays, waren wir in der Lage, die transversale Bewegung und die Verteilung von kurzkettigen, fluoreszenzmarkierten Phospholipidanaloga über beide Membranhälften in *ex vivo*-Membranen zu charakterisieren. Der stopped-flow BSA back-extraction Assay basiert auf der Technik der stopped-flow-Spektroskopie und der Tatsache, dass BSA in der Lage ist, kurzkettige, fluoreszenzmarkierte Lipidanaloga aus der äußeren Leaflet von (biologischen) Membranen zu extrahieren. Wir entschieden uns für invertierte Membranvesikel der Plasmamembran (IIMV) vom *E.coli* Wildtypstamm MG1655 als Untersuchungsobjekt, einerseits, weil diese Vesikel nur eine Membran besitzen und zum Anderen, weil IIMV sich sehr gut als Modell für den Flip-Flop von Phospholipiden nutzen lassen.

Wir beobachteten, dass kurzkettige, fluoreszenzmarkierte Analoga der beiden am häufigsten in *E.coli* vorkommenden Phospholipide, Phosphatidylethanolamin (PE) und Phosphatidylglycerol (PG), sehr schnell, d.h. mit Halbwertzeiten von weniger als drei Minuten, über die Membran von IIMV verteilten. Weiterhin verhielten sich kurzkettige, fluoreszenzmarkierte Analoga von den *E.coli*-fremden Phospholipiden, Phosphatidylcholin (PC) und Phosphatidylserin (PS), ähnlich wie die Analoga von PE und PG. Überraschenderweise, fanden wir heraus, dass alle oben genannten

Phospholipidanaloga im Gleichgewichtszustand nicht gleichmässig über beide Membranhälften verteilt waren. Unseren Ergebnissen zur Folge, waren nur ca. 23% der PE-, 18% der PC-, 34% der PG- und 26% der PS-Analoga auf der cytoplasmatischen Hälfte der IIMV lokalisiert. Schlussfolgernd aus unseren Analysen konnten wir zeigen, dass die transversale Bewegung von Phospholipidanaloga über die Membran von IIMV sehr schnell, bidirektional und kopfgruppenunbhängig ist. Diese Ergenisse konnten wir durch einen alternativen Assay, der auf der chemischen Löschung der Fluoreszenz durch Dithionit beruht, untermauern. Die Analyse von Proteoliposomen, die mit kopfgruppenmarkierten oder langkettigen, fluoreszenten Analoga von PE markiert waren, ergaben, dass die Kettenlänge von Phospholipidanaloga keinen Einfluß auf den Flip-Flop ausübt.

Inwiefern Proteine an dieser transversalen Bewegung der Phospholipidanaloga beteiligt sind, sollten Messungen des Flip-Flop von Analoga an unbehandelten und mit Proteinase K inkubierten Vesikeln zeigen, die aus einem Detergenzextrakt von IIMV rekonstituiert wurden. Zunächst konnten wir zeigen, dass die schnelle Bewegung der Phospholipidanaloga über die Membran von rekonstituierten, nicht mit Proteinase K behandelten Vesikeln (Proteoliposomen) erhalten blieb. Nach Inkubation mit Proteinase K wurde jedoch der Flip-Flop von PE- und PG-Analoga vollständig inhibiert. Um die Proteinabhängigkeit dieses Prozesses intensiver zu untersuchen, rekonstituierten wir Serien von Proteoliposomen mit ansteigendem bakteriellen Proteingehalt. Während wir in Proteoliposomen ohne bakterielle Proteine keinen Flip-Flop feststellen konnten, beobachteten wir, dass in Proteoliposomen mit mehr als 100 µg/ml Proteingehalt, der Flip-Flop der kurzkettigen Analoga von PE und PG sehr schnell war – mit Halbwertzeiten von weniger als zwei Minuten, die wir auch in isolierten IIMV fanden. In Proteoliposomen mit weniger als ~100 µg/ml korrelierte die Anzahl der durch BSA extrahierten fluoreszenten Analoga mit der Menge an aus IIMV in Proteoliposomen rekonstituierten Proteinen. Dieser Umstand wies eindeutig darauf hin, daß eine Proteinkonzentration von unter ~100 µg/ml in den Vesikeln nicht ausreicht, um jeden Vesikel mit einem Flippaseprotein auszustatten. Diese Daten zeigten sehr deutlich, dass die

transversale Bewegung von Phospholipiden über die innere Membran von *E.coli* durch Proteine vermittelt wird.

Zur Identifizierung der molekularen Grundlagen der proteinvermittelten, schnellen Transversalbewegung von Phospholipiden über IIMV-Membranen, nutzen wir Ionenaustauschchromatografie. Um die IIMV-Proteine zu separieren, wurden Detergenzextrakte von IIMV mittels eines starken Anionenaustauschers in zwei Fraktionen getrennt, und diese fraktionierten Proteine in Proteoliposomen rekonstituiert. Zur unserer Überraschung mussten wir feststellen, dass in keiner der rekonstituierten Fraktionen eine nennenswerte Anreicherung der Flippaseaktivität auftrat. Möglicherweise sind mehrere Proteine, mit unterschiedlichen Nettoladungen, oder aber auch Untereinheiten, die sich nicht durch Anionenaustauscher trennen liessen, am Flip-Flop von Phospholipiden beteiligt. Weitergehende Analysen mit anderen Proteinfraktionierungsmethoden sind notwendig, um den oder die Flippasekomplex(e) zu identifizieren.

Dennoch, die hier präsentierten Daten liefern starke Beweise für die Hypothese, dass der bidirektionale transversale Phospholipidaustausch zwischen den Membranhälften biogener Membranen proteinvermittelt und kopfgruppen-unabhängig ist und kein ATP benötigt.

9 Literature

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Publications

Marx, U., Lassmann, G., Holzhutter, H. G., Wüstner, D., Muller, P., Höhlig, A., Kubelt, J., Herrmann, A. (2000). Rapid flip-flop of phospholipids in endoplasmic reticulum membranes studied by a stopped-flow approach. *Biophysical Journal* **78**: 2628-2640

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Poster presentations

28th Steenbock Symposiums „Intracellular Protein and Lipid Traffic“, Madison, Wisconsin, USA

46th Annual Meeting of the Biophysical Society, 2002, San Francisco, California, USA

Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.

Berlin, den 06.01.2004

Janek Kubelt